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14. ABSTRACT

The work performed under this grant evaluated the efficacy of Glial Growth Factor 2 (GGF2), a member for the neuregulin family, as a spinal cord injury (SCI) therapeutic. The research expanded on previous work and extended these studies to determine if GGF2 should be advanced into human clinical studies for SCI. GGF2's optimal dosage was examined using a model of rodent SCI and testing several dose levels delivered either subcutaneously (SC) or intravenously (IV). Optimized dosing was determined to be by a SC route, and subsequent studies evaluated recovery of function by monitoring bladder function, muscle volume changes, and motor function in SCI rodent models of various severities. Additionally, safety and tolerability was explored in naïve canines, and followed by a clinical trial for safety in dogs with SCI.

15. SUBJECT TERMS Spinal Cord Injury, Glial Growth Factor 2, GGF2

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Introduction

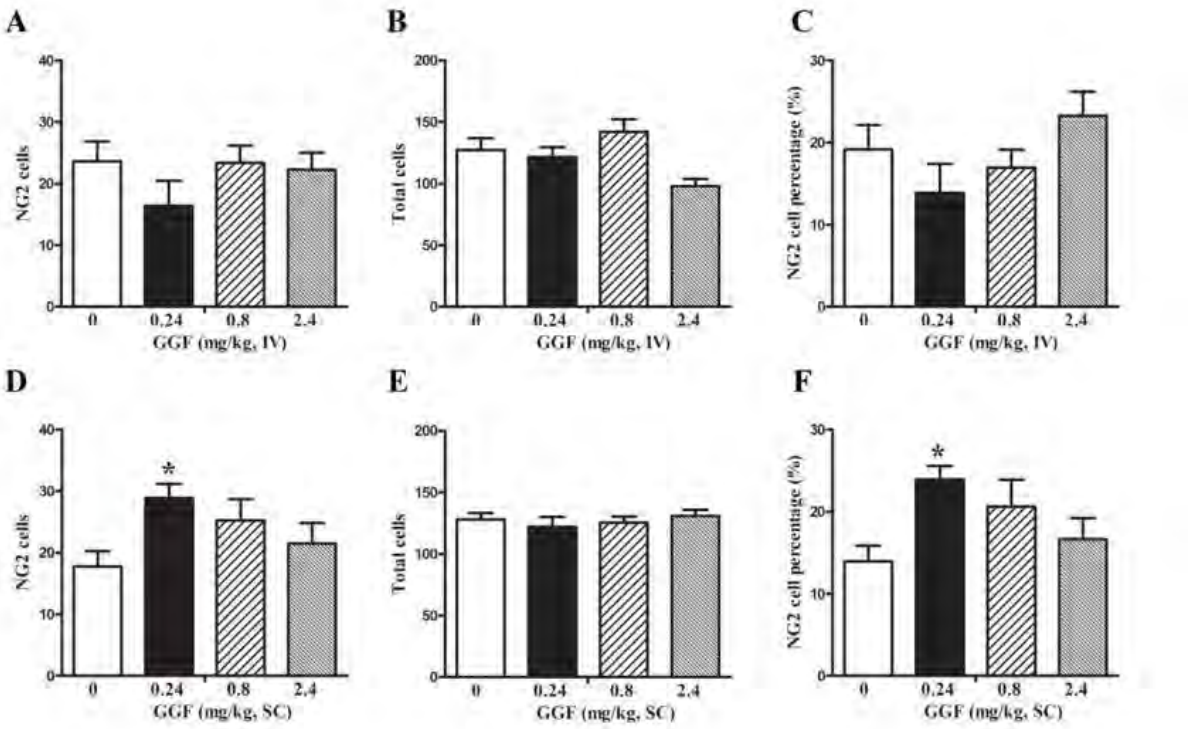
This grant funded a multi-institutional, multi-disciplinary partnership combining scientific, drug development and clinical expertise to evaluate recombinant human GGF2 for the treatment of spinal cord injury (SCI). GGF2 has shown great therapeutic potential in stroke and preliminary SCI models and the studies performed further evaluated the efficacy and safety of GGF2 in the SCI setting. There is a profound unmet need for the development of effective therapies for SCI in both the military and civilian setting. Glial Growth Factor 2 (GGF2) is a naturally occurring neuregulin that is essential for normal development of the nervous system (Lemke et al., 1996). Neuregulins promote recovery and restore function in multiple models of traumatic injuries including stroke (Iaci et al., 2010, Xu et al., 2005) and SCI (Whittaker, et al 2010). Additionally, GGF2 has been produced under GMPs and has completed GLP safety and toxicology demonstrating its suitability for experimental human use. GGF2 is currently in early phase trials for heart failure under the IND 106,077 (Acorda Therapeutics).

This multi-institutional partnership combined the laboratory SCI research expertise of Dr. Wrathall, the veterinary clinical expertise of Dr. Olby and the drug development and clinical expertise of Acorda Therapeutics. The studies under this grant assessed optimal dosing regimens in two different models of rodent SCI and evaluated the safety and tolerability of GGF2 in dogs with and without SCI. Three projects were pursued, including optimized dosing regimens in rodents with weight-drop SCI using a biomarker of oligodendrocyte proliferation, confirmation and extension of optimized dosing at 2 severity levels (severe and moderate) in a contusion model of rat SCI and finally exploration of safety and tolerability in a canine trial of naturally occurring SCI. The goal of the overall project was to provide strong evidence whether or not GGF2 should be advanced to human clinical studies for SCI.

Body

The purpose of this translational project is to identify the most appropriate dosing schedule and administration route of glial growth factor 2 (GGF2) in the treatment of rodent spinal cord injury (SCI) and to translate this work to dogs with naturally occurring SCI.

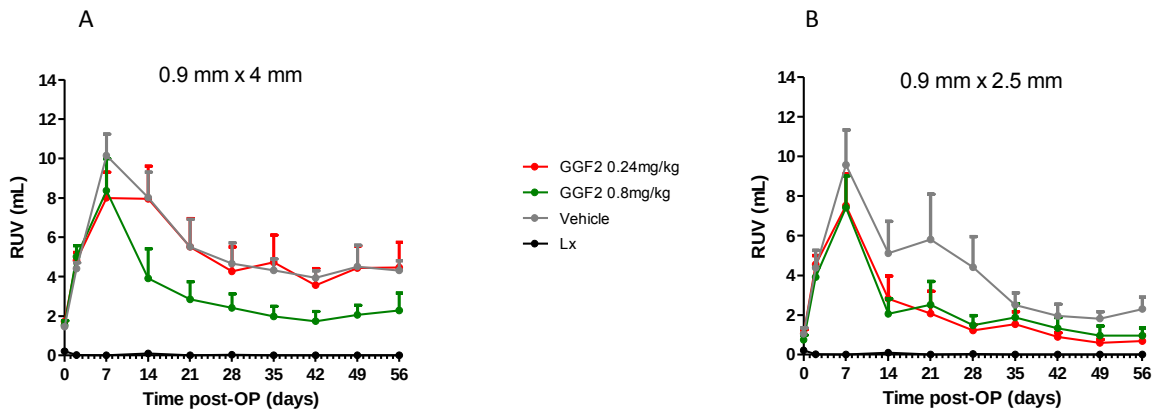
The goal of the studies in Year 1 was to identify the optimal dose and route of administration of GGF2 to increase NG2-expressing glia cells in residual white matter in a standardized rat model of contusion spinal cord injury (SCI). At 24 hours after injury, and daily thereafter for 1 week, rats (n = 8 per group) received injections of saline alone (0 GGF2) or saline containing the specified dose of GGF2 (0.24, 0.8, 2.4 mg/kg) either intravenously (IV) or subcutaneously (SC). As expected, there was no evidence of adverse effects in any treatment group on survival and no significant behavioral effect at one week after SCI. Quantitative analysis of immunohistochemically stained tissue sections showed that subcutaneous administration of the lowest concentration of GGF2 (0.24 mg/kg) significantly increased the number of NG2-expressing cells in residual ventral medial white matter 2-3 mm rostral and caudal to the injury epicenter from 14 to 24% of total cells. The intermediate dose (0.8 mg/kg) showed a tendency towards increased NG2+ cells but it was not statistically significant. Neither the SC high dose (2.4 mg/kg) nor any of the treatment doses administered IV, produced a significant effect on percent of NG2+ cells (or total NG2+ cells) in residual white matter at 1 week after injury, the chosen cellular biomarker we used for treatment effect (see Appendix 1: **SC100266P1 2012 Annual Report**).



One week of SC (D, E, F) but not IV (A, B, C) administration of GGF2 has a significant effect on NG2+ cells. SC administration of the lowest dose of GGF (0.24 mg/kg) significantly increases the NG2+ cells (D) and percentage of NG2+ cell percentage (F) in the spared white matter 2-3 mm from the epicenter of injured spinal cord at 1 week after SCI, compared to saline treated controls. ANOVA with Dunett's post hoc test, p<0.05.

Based on these results, the studies in year 2 compared effects of SC administration of 0.24 and 0.8 mg/kg GGF2 daily for 7 days on hind limb functional recovery over 6 weeks after SCI and on chronic histopathology, compared to vehicle treated animals as well as animals receiving no treatment. This study did not detect a difference between treatment groups, but further histology revealed that the model, which historically results in left/right symmetry of the contusion injury had a disproportionate number of animals with asymmetrical injuries. This may have confounded the efficacy results (see Appendix 2: **SC100266 Wrathall Year 2 Progress report).** This work and the studies from Year 1 were completed at Georgetown University.

Concurrent to the above study, dosing studies were further expanded on in year 2 using a forceps compression model of rat SCI at 2 injury severities, and included hind limb functional endpoints as well as bladder function and skeletal muscle volume measures. GGF2 when dosed at 24 h post-injury produced significant improvements in locomotor function as assessed by the BBB locomotor rating scale following both severe and moderate compression SCI. Bladder function also improved with treatment at both injury severities, and there was no difference in skeletal muscle volumes. A follow on study compared dosing initiated at 24 hours post SCI with and without continued maintenance dosing twice a week for 9 weeks following the first week of daily dosing. In this second study only modest improvements in function were observed in all treated animals, and were not statistically different from vehicle(see Appendix 3: **SC100266 2013 Annual Report**). This work was completed at Acorda Therapeutics.



Effects of GGF2 on bladder function (RUVs) following severe (A) and moderate (B) SCI. Data are presented as mean ± SEM. A. $p<0.05$ GGF2 0.8 mg/mL vs vehicle. B. $p<0.05$ GGF2 at both doses vs vehicle (repeated measures ANOVA).

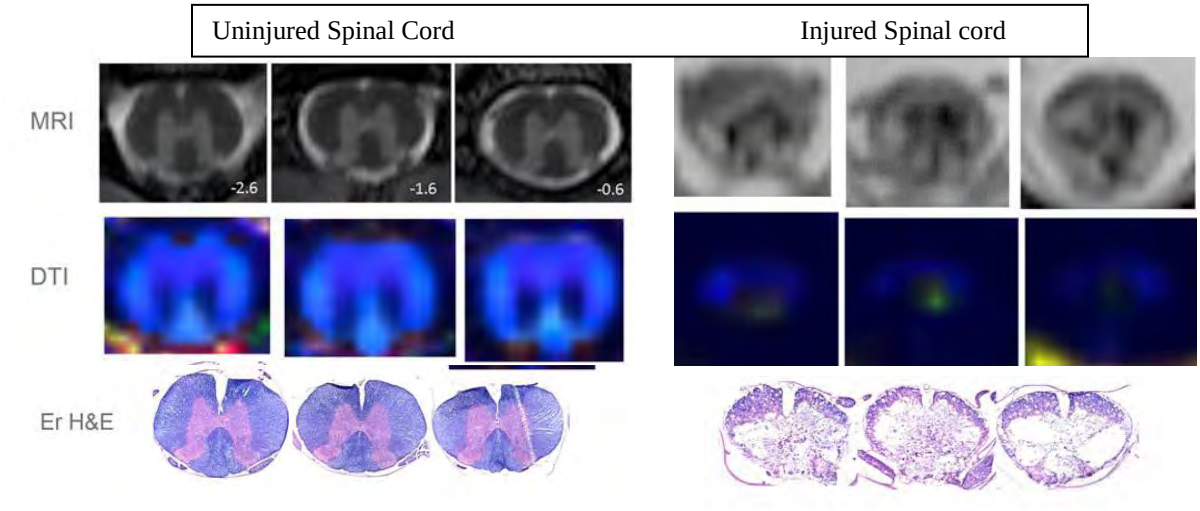
Evaluation of spinal cord sections from the above studies stained with hematoxylin/eosin confirmed that spinal cord injury in rats was successfully induced in both the vehicle and GGF2-treated groups compared to sham-operated animals. Immunohistochemical analysis 9 weeks after injury demonstrated that neither GAP43 nor synaptophysin expression was statistically different between GGF2 and vehicle treatment at levels at or surrounding the compression lesion. BDNF expression was generally elevated at 9 weeks at and around the compression zone following spinal cord injury and was reduced toward sham levels by GGF2 treatment rostral to the lesion.

Ultrasonographic assessment of peripheral muscles (gastrocnemius) distal to the lesion showed that GGF2 treatment did not improve skeletal muscle gross morphology. Histologic data showed that GGF2 improved interstitial fibrosis and myofiber size in atrophied gastrocnemius muscle following SCI; however, other studies from our laboratory in denervated rat gastrocnemius muscle show that this effect is likely due to direct effects on skeletal muscle. The rat studies demonstrated that GGF2 did produce a response in SCI animals, but it was not clear how robust of a response this was.

Taken together, the Georgetown University and Acorda studies show that systemic administration of GGF2 produced variable effects on locomotor and bladder functional recovery in rats with spinal cord injury.

To complete the rat studies, in year 3 of this collaborative project we compared histopathological data from SCI rat tissue from the Georgetown and Acorda models of incomplete thoracic injury to see similarities and differences in these rodent SCI models. Comparative mid-lesion images from 2 representative rats that exhibited similar chronic functional recovery (BBB = 12 at 6-8 weeks) were analyzed. The Georgetown SCI lesion was characterized by central cavities with variable but limited cells within the central aspect of the lesion. A peripheral rim of partial preserved myelinated white matter was most evident ventrally and ventral-laterally. The lesion in the Acorda model was characterized by a dorsal-ventral flattening of the cord profile with a highly cellular central lesion area and few cavities. The peripheral rim of preserved white matter tended to be thickest in the dorsal aspects of the cord.

In order to develop an imaging endpoint that correlates with spinal cord injury and recovery following therapeutic intervention, representative injured rat spinal cords from the Georgetown model were evaluated by MRI/Diffusion Tensor Imaging at Georgetown. The DTI images revealed the loss of white matter at and near the injury epicenter and, as shown below, reflected the histopathology seen in stained tissue sections. A number of different imaging conditions were evaluated to compare DTI results with qualitative and quantitative measures of histopathology as well as functional impairment and overall white matter sparing (see Appendix 2: SC100266 Wrathall Year 2 Progress report and Appendix 4 SC100266 Wrathall Year 3 Progress report).



At the center of the lesion, using the entire spinal cord cross section as the ROI, fractional anisotropy (FA mean) as well as the FA ID (corrected for the area of the injured spinal cord) were significantly lower in each of the injured spinal cords as compared to the uninjured controls. However, the differences in these two measures among SCI rats did not seem to be related to each other or distinguish between rats with differences in functional recovery (BBB) or differences in the histopathology of the injury sites.

Rat #	BBB	FA mean	FA ID
Uninjured 1	21	0.63	90
721	8	0.23	9
725	8	0.25	17
705	12	0.31	20
727	13	0.20	20
709	19	0.24	18

Dog studies were initiated after the 3rd rat study demonstrated successful functional recovery. This PK and tolerability study in healthy dogs demonstrated safe dosing with 1 week of initial treatment, but animals developed hypersensitivity with re-treatment following 1 month of washout. A revised statement of work was approved to further explore the tolerability of GGF2 in dogs with and without SCI in year 3. GGF2 was administered SC (0.08mg/kg) to 10 dogs once a day for 7 days; 5 dogs had intrathecal catheters placed for 6 hours to mimic previous experimental conditions. An equal volume of vehicle was administered in the same way to 5 additional dogs. Adverse effects were not detected in any of the dogs during administration or the following 3 months. Based on this, 10 dogs with naturally occurring spinal cord injuries due to disc herniations were recruited to a phase 1 safety trial. Dogs

entering the study were paraplegic with no pain perception and presented within 24 hours of onset of paralysis. All underwent advanced imaging to identify their herniated disc and were surgically decompressed. Dogs were randomized to receive GGF2 or vehicle at a dose rate (or equivalent volume) of 0.08mg/kg SC once a day for 7 days. Treatment was initiated as soon as the diagnosis was confirmed. Presence of adverse effects and recovery were monitored daily during the 7 days of postoperative hospitalization, and then at 2,4 and 8 weeks postoperatively, when an MRI was repeated. There were no adverse effects noted in any of the dogs during GGF2 administration. Overall, 2 dogs in each treatment group failed to recover motor function or sensation with all remaining dogs showing a robust recovery. We conclude a single course of 7 days of once daily SC GGF2 is safe in dogs with acute spinal cord injury. Efficacy has not yet been evaluated (see appendix 5: SC100266 Olby Final report)

Key Research Accomplishments

- Rigorously tested effects of GGF2 dosing regimens in multiple models of SCI in independent laboratories, demonstrating positive effects histologically and functionally, though with variable robustness of effect.
- Demonstrated safety and tolerability of GGF2 in naïve and SCI dogs when delivered systemically.

Reportable Outcomes

The investigators concluded that these data on their own do not support further development of GGF2 for the treatment of spinal cord injury.

Conclusion

The investigators conclude that while it appears GGF2 treatment is in some way impacting outcomes following SCI, the results were variable and not robustly reproducible. In the rat weight-drop SCI studies at Georgetown, the first study demonstrated outcomes consistent with previous data, where increased NG2 positive cells were seen with daily dosing for 1 week following SCI. When the second study was undertaken to expand on these outcomes in the same model with functional outcomes at 6 weeks post injury, the data was neutral with respect to improved function. In the concurrent studies at Acorda with the foreceps compression rat model of SCI, GGF2 treatment resulted in robust functional improvements in both the severe and moderate injury groups. However, the second study at Acorda, evaluating 1 week of daily dosing with and without continued twice weekly dosing failed to result in as robust a response in walking and bladder improvements. Dog PK and tolerability studies demonstrated that hypersensitivity develops in uninjured dogs exposed a second time to GGF2 at one month following the initial 1 week of daily dosing. The follow on clinical trial included just the 1 week of daily subcutaneous dosing which was safe and well tolerate in dogs with spinal cord injury. Despite the safety profile in larger animal SCI, the lack of a truly robust recovery in all models tested in this proposal does not provide strong evidence that GGF2 should continue to be developed for SCI at this time.

This work represents a very well planned and executed development strategy for the translational evaluation of a therapeutic, using experienced researchers in the field to test the effects of a drug

candidate in multiple models. While these results do not indicate that further development is warranted at this time, the projects were executed in a timely and scientifically rigorous manner.

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Whittaker MT, Zai LJ, Lee HJ, Pajoohesh-Ganji A, Wu J, Sharp A, Wyse R, Wrathall JR. GGF2 (Nrg1-β3) treatment enhances NG2+ cell response and improves functional recovery after spinal cord injury Glia. 2012 Feb; 60(2):281-94.

Appendix 1: SC100266P1 2012 Annual Report

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14. ABSTRACT
The goal of the studies in Year 1 was to identify the optimal dose and route of administration of GGF2 to increase NG2-expressing glia cells in residual white matter in a standardized rat model of contusion spinal cord injury (SCI). On the first day after SCI, rats were behaviorally tested to confirm expected symmetrical functional deficits and randomly assigned to treatment groups using a randomized block design. At 24 hours after injury, and daily thereafter for 1 week, rats (n = 8 per group) received injections of saline alone (0 GGF2) or saline containing the specified dose of GGF2 (0.24, 0.8, 2.4 mg/kg) either intravenously (IV) or subcutaneously (SC). As injections were made from vials provided by Acorda, designated A, B, C, D for IV administration, or W, X, Y and Z, for SC administration, the investigators at Georgetown were blind to the treatment group assignment. As expected, there was no evidence of adverse effects in any treatment group on survival and no significant behavioral effect at one week after SCI. Quantitative analysis of immunohistochemically stained tissue sections showed that subcutaneous administration of the lowest concentration of GGF2 (0.24 mg/kg) significantly increased the number of NG2-expressing cells in residual ventral medial white matter 2-3 mm rostral and caudal to the injury epicenter from 14 to 24% of total cells. The intermediate dose (0.8 mg/kg) showed a tendency towards increased NG2+ cells but it was not statistically significant. Neither the SC high dose (2.4 mg/kg) nor any of the treatment doses administered IV, produced a significant effect on percent of NG2+ cells (or total NG2+ cells) in residual white matter at 1 week after injury, the chosen cellular biomarker we used for treatment effect. Based on these results, in year 2 we propose to compare effects of SC administration of 0.24 and 0.8 mg/kg GGF2 daily for 7 days on hind limb functional recovery over 6 weeks after SCI and on chronic histopathology. We will compare outcome after these treatments to that after SC injections of vehicle (saline) alone, and to SCI with no treatment.

15. SUBJECT TERMS Spinal Cord Injury, Glial Growth Factor 2, GGF2

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Introduction

Administration of GGF2 (0.8-1mg/kg) beginning at 24 h after SCI and continuing once daily for 1 week increases the proliferation of NG2-expressing glial progenitor cells after SCI in rat and mouse models (Zai, 2005; Whittaker, 2012). Both IV and SC routes may be required for acute and then longer dosing in humans. To facilitate translation of GGF2 treatment for SCI we planned to compare IV to SC GGF2 administration and performed a dose response study. We compared the increases in NG2 (a marker of oligodendrocyte precursors and pericytes) expressing cells in the spared white matter in the injured spinal cord after the one-week treatment period, since our previous studies and preliminary results showed that this is the initial effect of GGF2 treatment after SCI (Zai, 2005; Lytle et al., 2009; Whittaker, 2010) that appears to predict long-term beneficial effects on functional recovery chronically after SCI.

Methods

Young adult female Sprague Dawley rats (approximately 250g) were anesthetized with chloral hydrate (360mg/kg). A laminectomy was performed at the eighth thoracic vertebra, opening an area approximately 2.8 mm in diameter. Spinal cord injury (SCI) was produced with a weight drop injury device (Wrathall, 1985) in which a plastic impounder (2.4 mm diameter tip) was lowered onto the exposed dura. A 10 g weight was dropped from a height of 2.5 cm onto the impounder device. The impact resulted in complete hind limb paralysis at 24 h after injury and long-term incomplete recovery of hind limb function. After SCI, rats are kept on highly absorbent bedding and their bladders are expressed twice daily before regaining normal function.

Behavioral Testing

Rat hind limb locomotor recovery was assessed at 1 day post-injury and 1 week after spinal cord injury using the Basso, Beatty, and Bresnehan (BBB) open field expanded locomotor score where animals with complete hind limb paralysis are scored 0, and animals with normal locomotion are scored 21 (Basso et al., 1995). Rats were also scored on a battery of tests to determine overall recovery of hind limb motor and sensory function using the Combined Behavioral Score [CBS (Gale et al.,1985)]. The CBS ranges from 0 for normal rats to 100 for those with complete hind limb paralysis that are abnormal on all of the tests.

GGF treatment

On the first day after injury, after behavioral testing to assure symmetrical functional deficit, rats were randomly assigned to receive injections from vials provided by Acorda and labeled A, B, C, D, for IV administration, and W, X, Y, Z for SC administration. The contents of these vials, to which the Georgetown investigators were blind, provided dosages 0, 0.24, 0.8, 2.4 mg/kg GGF2 dissolved in sterile saline. IV injections were through the tail vein and SC injections were below the skin of covering the back of the hip, on alternating sides of the animal.

Perfusion and Preparation of Tissue for Histopathology

One week after injury, after the last behavioral testing, subjects were anesthetized and transcardially perfused with phosphate buffered saline (PBS) followed by 4% buffered paraformaldehyde. Spinal cords were removed and segments centered on the injury epicenter were post-fixed, cryoprotected, and frozen embedded in OCT compound (Tissue-Tek). Stereological sets of 20 μm coronal sections were

prepared on a cryostat and representative slides were stained with eriochrome-cyanine to label myelin (Grossman et al., 2001) in order to assess tissue morphology and to determine the locations of the injury epicenter.

Immunohistochemistry and Quantitative Analysis

Immunohistochemistry was performed on spinal cord sections at specified distances (2-3 mm) rostral and caudal to the injury epicenter using antibody to NG2 (rabbit anti-NG2, Millipore) with immunoperoxidase (DAB) detection using an ABC kit (Vector).

NG2+ cells were counted with bright-field microscopy at 100x using a Zeiss Axioplan 2 microscope within a region of interest (ROI) of 0.0144mm2 positioned in the left and right ventromedial areas of residual white matter. Cells were counted in two, 20µm cross-sections per animal located 2-3mm rostral and caudal to the epicenter (location of minimum preserved white matter). Representative sections were a minimum of 200µm and a maximum of 800µm apart. The mean number of NG2+ cells, the percentage of total cells that were NG2+, and the mean number of total cells in spared white matter for each subject is therefore determined from cell counts at eight separate ROI's. NG2+ cells of any intensity were counted if a cresyl violet-counterstained nucleus was observed. Only cell profiles within the reticule or touching the right or bottom lines were counted.

Sampling and statistical analysis

In all cases, the number of subjects (rats) served as the sample size. Data were reported as mean ± SEM. Significance was generally determined using one way ANOVA. If an overall significant effect of treatment was detected, Dunnett's post hoc test was used to assess the differences between groups. Significance was set at $P < 0.05$. Statistical analysis was carried out using SPSS.

Key research accomplishments

1. We performed 79 SCI surgeries on Sprague-Dawley female rats for Aim 1 of the GGF2 project. All rats were examined on the first day after SCI to determine whether their BBB score was 0 or 1 for both hind limbs. A total of 5 rats were eliminated for failure to meet this criterion. The remaining 74 rats were randomly assigned to treatment groups A, B, C, D (iv treatments) or W, X, Y, Z (sc treatments).

Two rats missed at least one planned drug treatment and were eliminated from the study. Two rats (in 2 different experimental groups) died prior to completing treatment. Their autopsies showed kidney infections, historically the major cause of death in experimental rodent SCI models. The remaining 70 rats were behavioral tested on day 7 and perfused with fixative for histopathological examination. Figure 1A (next page) shows the mean +/- SEM BBB (open field hind limb locomotion) and CBS (combined hind limb sensory-motor deficit) scores at 1 week after SCI for the different treatment groups (n = 8-11) for these 70 rats. Statistical analysis (one way ANOVA) showed no significance difference among the IV or SC groups at 1 week after SCI with either the BBB or CBS measures of hind limb function/deficit.

2. We serially sectioned spinal cord tissue centered in the epicenter for these 70 rats and completed analysis of their basic lesion histopathology. There were technical difficulties with some of the early tissue samples such that the tissue was damaged during dissection or embedding. Additional rats were generated to make up the desired group size of n = 8. The 1 week behavioral results for these 64 animals are shown in Figure 1B. As expected, there was no behavioral indication of a treatment effect this early,

at 1 week after SCI. ANOVA showed no difference between the groups in either the BBB or CBS. Tissue from these 64 rats was used to determine whether there was an effect of treatment group on the chosen biomarker, an increase in NG2+ cells in residual white matter at one week after SCI.

3. An important result from the study to this point was the absence of any detected adverse effect of treatment on survival or recovery at 1 week after SCI. Our mortality rate was extremely low (2 of 74 total rats or 2.7%) suggesting that even the highest dose is acceptable in the first week after SCI.

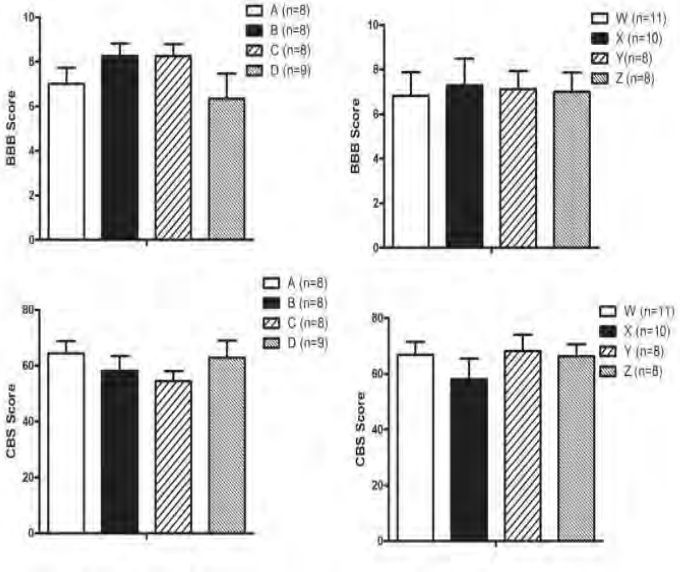

Fig A

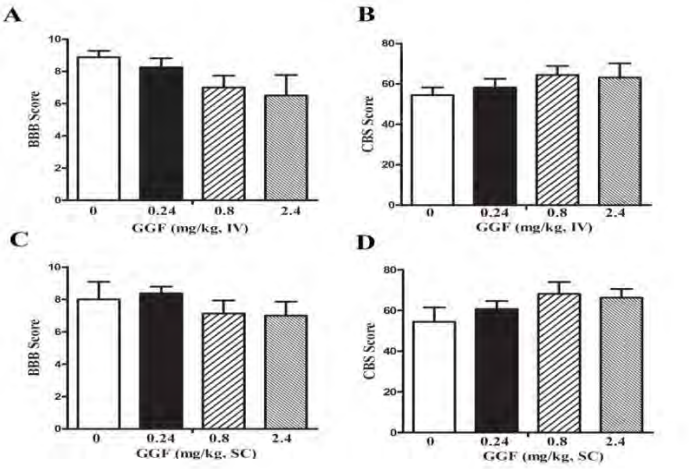

Fig B

Figure 1. *Behavioral evaluation of hind limb function at one week after SCI with administration of blinded treatment solutions daily by IV (groups A-D) or SC (groups W-Z) routes. Neither the BBB scale measuring open field locomotion, nor the CBS, evaluating overall hind limb sensorimotor function, indicated any significant difference among the groups. A. Results from the total of 70 SCI rats that completed the 1 week protocol. B. Results from the 64 rats that completed the protocol and whose tissue was acceptable for quantitative analysis of NG2+ cells.*

4. We established the injury epicenters by examining tissue sections stained with eriochrome –cyanine, as illustrated in Figure 2A. Sections located at 2-3 mm rostral and caudal to each epicenter were immunohistochemically stained for NG2 and numbers of positive cells determined in ventral medial

white matter (VMWM) RO1, as shown in Figure 2B. As expected from our previous studies (Lytle et al,2009; Whittaker et al., 2012), some of the NG2+ cells clearly expressed polydendrocyte morphology and were associated with axons (Figure 2E), consistent with their being oligodendrocyte precursor cells. Others were associated with blood vessels as expected for NG2+ pericytes (Figure 2D).

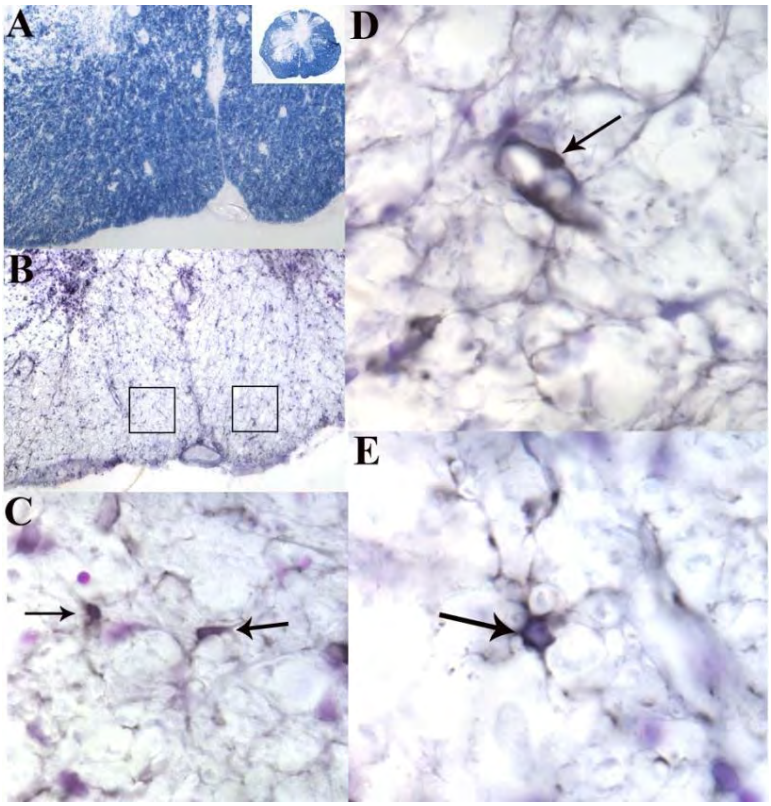

Figure 2: Ng2+ cells in residual ventral medial white matter (VMWM) at 2-3mm rostral or caudal to the epicenter in Aim 1 Study rats at 1 week after SCI. A. Low magnification view of VMWM (X10) and insert of (X 2.5) by eriochrome cyanine stain. B, Low magnification view of NG2 stain in region of interest inVMWM (X10). D-E. Cells photographed under high magnification (X40 for C and X100 for D and E) showing NG2+ cells (arrow) that have morphological features of oligodendrocyte precursors (E) and pericytes (D).

5. We counted NG2+ cells and total cells in the VMWM ROI and compared the results among the 4 groups of IV and the 4 groups of SC treated animals. After the counts were completed and the data sent to Acorda, we received the key to the blinded vials and were able to generate the charts shown in Figure 3 for NG2+ cells and NG2+ cells expressed as a percentage of total cells (to control for likely variations in injury severity).

Reportable Outcomes

We found no significant difference in total cells, NG2+ cells, or NG2+ cells expressed as a percentage of total cells among the groups that received IV GG2 treatment (Figure 3, A-C).

We found no significant difference in total cells among the groups that received SC GGF2 treatment (Figure 3, E). However, both NG2+ cells and NG2 cells expressed as a percentage of total cells were significantly greater in the group that received daily SC treatment with 0.24 mg/kg GGF2 than that in saline-treated controls (Figure 3, D, F). There was no apparent effect of treatment with the highest dose, 2.4 mg/kg. However, the intermediate dose, 0.8mg/kg, showed a tendency towards increase that was not statistically significant. A second, independent member of the staff also counted NG2+ and total cells in these samples and obtained similar patterns but also greater variability that prevented statistical significance. However, when the data from both investigators was combined and analyzed, the significant effect of treatment with 0.24mg/kg GGF2 was seen.

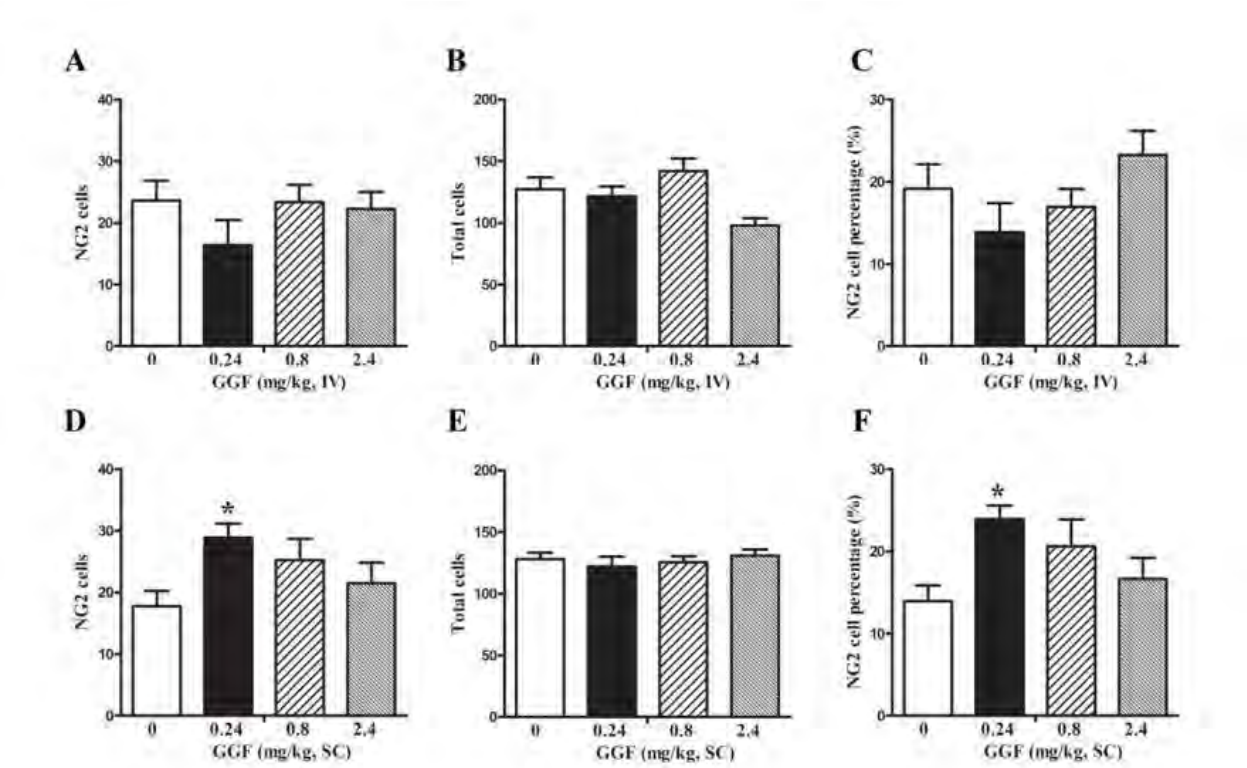

Figure 3. One week of SC (D, E, F) but not IV (A, B, C) administration of GGF2 has a significant effect on NG2+ cells. SC administration of the lowest dose of GGF (0.24 mg/kg) significantly increases the NG2+ cells (D) and percentage of NG2+ cell percentage (F) in the spared white matter 2-3 mm from the epicenter of injured spinal cord at 1 week after SCI, compared to saline treated controls. ANOVA with Dunett's post hoc test, $p<0.05$.

Conclusions

The results indicate that the best route of administration of GGF2 to increase NG2+ cells after SCI is subcutaneously. The best dosage tested is 0.24 mg/kg, lower than previously examined. However, 0.8mg/kg may also be effective, as has been reported previously (Lytle et al, 2009; Whittaker et al, 2012) possibly more so for less severe SCI.

Proposed Year 2 Studies Based on These Results:

As we found no effect of treatment administered IV, we propose to focus on the SC route of GGF2 administration. We will compare 4 groups of rats over 6 weeks after SCI to determine effects of

treatments on long-term recovery of hind limb function and on chronic histopathology, as described in our original proposal. As in the Aim 1 Study, at 24 h after SCI rats will be randomly assigned to blinded treatment with coded vials containing GGF2 at a concentration to provide 0.24, 0.8 or 0 mg/kg daily, or to serve as no treatment SCI controls. A sample size of 12 rats in each group will be used, as specified in the original approved proposal.

We will also investigate the use of DTI to evaluate effects of treatment on white matter integrity after SCI, as detailed in our original approved proposal.

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Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma. 1995 Feb;12(1):1-21.

Gale K, Kerasidis H, Wrathall JR. Spinal cord contusion in the rat: behavioral analysis of functional neurologic impairment. Exp Neurol. 1985 Apr;88(1):123-34.

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Appendix 2: SC100266 Wrathall Year 2 Progress report

SC100266P1 - "Translational Pharmacologic Efficacy Studies of Glial Growth Factor 2 (GGF2) in Spinal Cord Injury Models and in the Veterinary Clinical Setting"

Georgetown University Year 2 Progress Report – October, 2013

Jean R. Wrathall, PhD (Principal Investigator), Xinchun Jin, MD, PhD (Postdoctoral Fellow), Alexandra Palmisano, BS (Research Assistant

Project 1, Aim 2. The goals of our studies in year 2 were to (a) use the most effective dosages and route of administration of GGF2 on the proliferation of NG2-expressing glia cells after SCI that we identified in year 1 in long term studies of effects on functional recovery and chronic histopathology and (b) subject spinal cords from some of these rats to MRI and Diffusion Tensor Imaging (DTI) of the spinal cord to develop MRI imaging method that can detect treatment effects that improve survival and myelination of axons after SCI for potential use in future dog studies.

Methods Young adult male and female Sprague Dawley rats (approximately 250g) were anesthetized with chloral hydrate (360mg/kg). A laminectomy was performed at the eighth thoracic vertebra, opening an area approximately 2.8 mm in diameter. Spinal cord injury (SCI) was produced with a weight drop injury device (Wrathall, 1985) in which a plastic impounder (2.4 mm diameter tip) was lowered onto the exposed dura. A 10 g weight was dropped from a height of 2.5 cm onto the impounder device. After SCI, rats are kept on highly absorbent bedding and their bladders are expressed twice daily before regaining normal function. Urine was collected and the volume recorded to assess the time course of recovery of spontaneous voiding.

On the first day after SCI, rats were behaviorally tested to confirm functional deficits and assigned to treatment groups using a randomized block design. At 24 hours after injury and daily thereafter for 1 week, rats (n = 12 per group) received no injection (SCI controls) or injections of saline alone (0 GGF2) or saline containing the specified dose of GGF2 (0.24, 0.8 mg/kg) subcutaneously (SC). Injections were made from vials provided by Acorda, designated F, G or H: the investigators at Georgetown were blind to the treatment group assignment for injected animals until the study was completed.

Behavioral Testing

Rat hind limb locomotor recovery was assessed at 1 day post-injury and weekly thereafter using the Basso, Beatty, and Bresnahan (BBB) open field locomotor score where animals with complete hind limb paralysis are scored 0, and animals with normal locomotion are scored 21. Rats were also scored on a battery of tests to determine overall recovery of hind limb motor and sensory function using the Combined Behavioral Score [CBS (Gale et al.,1985)]. The CBS ranges from 0 for normal rats to 100 for those with complete hind limb paralysis that are abnormal on all of the tests.

Perfusion and Preparation of Tissue for MRI Imaging and Histopathology

Six week after injury, after the last behavioral testing, subjects were anesthetized and transcardially perfused with phosphate buffered saline (PBS) followed by 4% buffered paraformaldehyde. Spinal columns were removed and stored in 10% sucrose for MRI imaging after which a 1.5 cm cord segment centered on the injury epicenter was cryoprotected and frozen embedded in OCT compound (Tissue-Tek). Serial 20 µm coronal sections were prepared on a cryostat. Representative slides for each mm of tissue were stained with eriochrome-cyanine to label myelin in order to assess tissue morphology and to determine the locations of the injury epicenter.

MRI and DTI Imaging.

Ex-vivo rat spinal columns immersed in 10% sucrose underwent MR imaging at the Preclinical Imaging Research Laboratory (PIRL) of the Lombardi Cancer Center at Georgetown University. The MR imager used is a 7.0 Tesla horizontal Bruker magnet with a 20 cm bore equipped with 100 gauss/cm microimaging gradients and run by Paravision 5.0 software. The samples were imaged in a 40 mm transmit-receive volume RF coil. To acquire anatomical images, a two-dimensional T2–weighted Turbo RARE (rapid acquisition with rapid enhancement) protocol was used with 30 interlaced slices in the axial plane, TR: 4235 ms, TE: 36 ms, RARE factor: 6, averages: 4, field of view: 25 X 25 mm, matrix: 256 x 256, and slice thickness of 1 mm. Subsequently, two-dimensional DTI was performed with TR: 12500 ms, TE: 21.1ms, FA: 90 deg, eff BW: 300000 Hz, segments: 4, averages: 8, diffusion directions: 30, b: 1900s/mm^2, slice thickness: 1mm, FOV: 25 mm x 25 mmm. Fractional anisotropy of white and gray matter in the ex-vixo thoracic spinal cord of the injured versus un-injured subjects was measured in selected regions of interest (ROI).

Immunohistochemistry and Quantitative Analysis

Immunohistochemistry was performed on spinal cord cryostat sections from MRI-imaged tissue at specified distances – the injury epicenter and 1 mm rostral and caudal to the injury epicenter - using antibody to axons (NF200) and both central (PLP) and peripheral (PO) myelin. Fluorescent secondary antibodies were used to detect immunostaining and images were obtained from a Zeiss fluorescent microscope. Analysis of fractional percent of tissue that was immunostained in specified regions of interest was performed using NIH Image J.

Sampling and statistical analysis

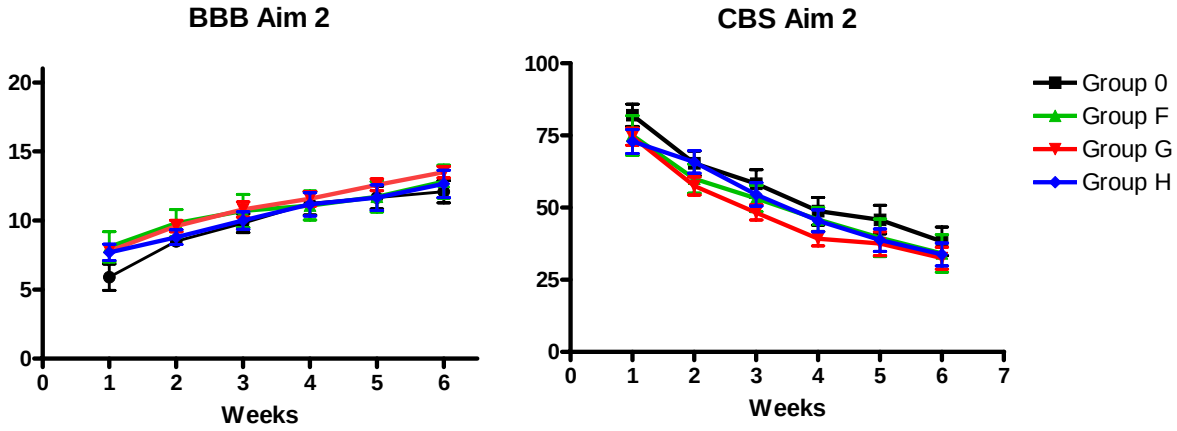
In all cases, the number of subjects (rats) served as the sample size. Data were reported as mean ± SEM. Significance of behavioral results was determined using repeat measures ANOVA. If an overall significant effect of treatment was detected, Dunnett's post hoc test was used to assess the differences between groups. Other data were evaluated with Student's t-test (2 groups) or one way ANOVA (3 or more groups). Significance was set at $P < 0.05$. Statistical analysis was carried out using SPSS.

Research accomplishments

Project 1, Aim 2a. We performed SCI on 52 young adult Sprague Dawley male and female rats. All survived surgery and were examined behavioral with the BBB test of hind limb locomotion on the first day after injury. All but 2 received a 0 or 1 BBB score on each hind limb and were accepted into the study. These rats were randomly assigned to receive either no treatment (0 group) or to begin at 24 h after SCI daily s.c. injections from the treatment vials F, G or H through day 7 after injury. The rats for each weeks' surgery (total n = 16) were considered an experimental block and randomized to 4 each (2 male, 2 female) in each of the 4 experimental groups (0, F, G, H). Bladders were manual expressed twice daily until a reliable reflex bladder was established and rats were behaviorally tested weekly for recovery of hind limb function using both the BBB measure of locomotion and the CBS measure of overall hind limb function. At 6 weeks after the last behavioral test, each rat was perfused with fixative for potential MRI DTI (diffusion tensor imaging) to evaluate integrity of white matter tracts and subsequent analysis of spinal cord histopathology and immunohistochemnistry.

All rats in groups 0, G and H survived the 6 week study for a final n = 12. Three rats in group F died prematurely, all with bladder/kidney infections that we were unable to effectively treat with antibiotic. We replaced 2 of these but the third died late in the study and was not replaced so that the final n in the F group = 11 rats.

Figure 1 (below) shows the results from behavioral testing of hind limb function using the BBB and the CBS. Normal rats have a BBB score of 21 and a CBS score of 0.

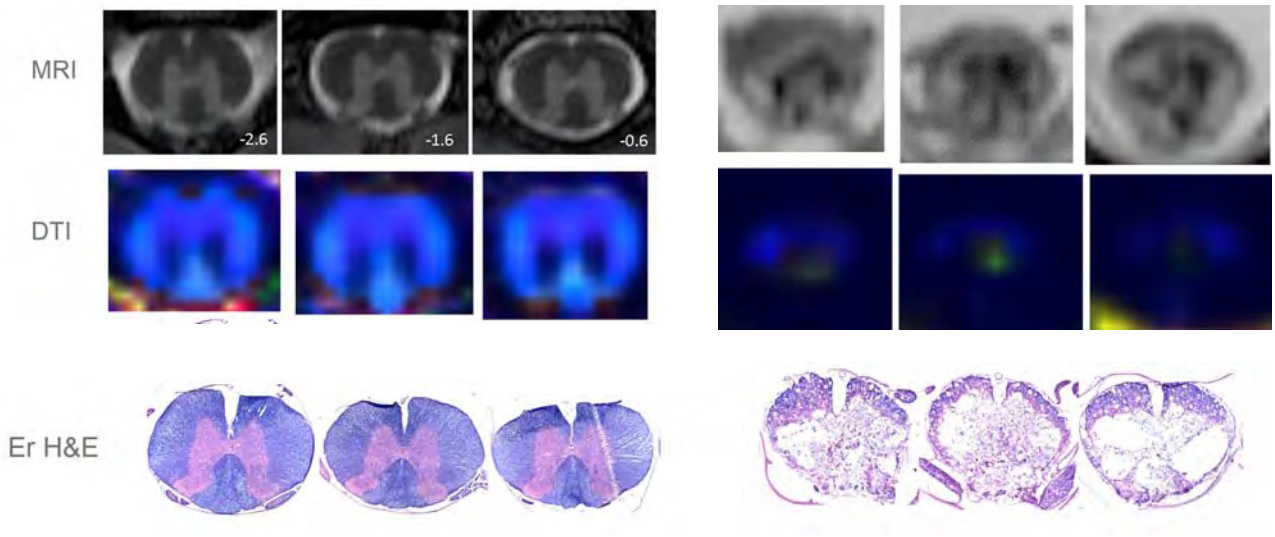


Repeat measures ANOVA detected no significant difference in recovery among the 4 groups. Additional comparisons of male and female rats in the study (there were approximately equal numbers of each) showed no significant difference overall or in comparing the two sexes in different treatment groups. In addition we compared recovery of spontaneous micturition (voiding) and saw no group effect on when this occurred.

Project 1, Aim 2a. Given these results we decided to use tissue from the control groups in this study and additional uninjured rats to attempt to correlate MRI and DTI results with detailed histopathology chronically after SCI, while we awaited results from our collaborators at Acorda from their independent tests of GGF2 efficacy in rat models of SCI.

The key to the blinded injection vials was obtained from Acorda; F = 0.8mg/ml solution, G = 0.24 mg/kg and H = vehicle. Rats from the H group and from the 0 treatment group were used for the Aim 2a study to represent SCI rats independent of any effect of GGF2. Rats from these two groups were selected with high, medium and low functional recovery to provide what we expected to be a wide range of histopathology and thus approximate a "standard curve" of injury severity. Spinal cords in their vertebral columns were imaged in the Small Animal Imaging Center at Georgetown University Medical Center and then the spinal cords dissected for cryostat sectioning, routine histopathology and evaluation of white matter sparing and for immunohistochemical analysis of axonal and myelin staining.

After establishing the best imaging parameters, a total of 10 chronic SCI rat cords (6 weeks after injury) and 5 from uninjured age matched controls were evaluated. Figure 2, below, shows examples of results from one of the uninjured controls compared to a SCI rat (rat 721) where the injury epicenter and tissue 1 mm rostral and caudal to it is shown with anatomical MRI, DTI and histopathology (sections stained with eriochrome and H& E counterstain).



Uninjured Control SCI Rat 721

Adjacent tissue sections representing the epicenter and 1 mm rostral and caudal to it were stained with antibodies to axons (NF200) and myelin (PLP for CNS myelin and PO for peripheral myelin) and fluorescent images obtained in specific regions of interest (ROI) including ventral medial white matter, lateral white matter, dorsal white matter and the central cord. Figure 3, shows examples of fluorescent images of the NF200 and PLP immunoreactivity in the ventral medial and ventral lateral ROI from the control and SCI Rat 721 above. (Note that in

Figure 2 the cord sections are shown with the ventral side as in the MRI images while in Figure 3 they are reversed and shown ventral side down, as typical in rodent spinal cord injury studies.)

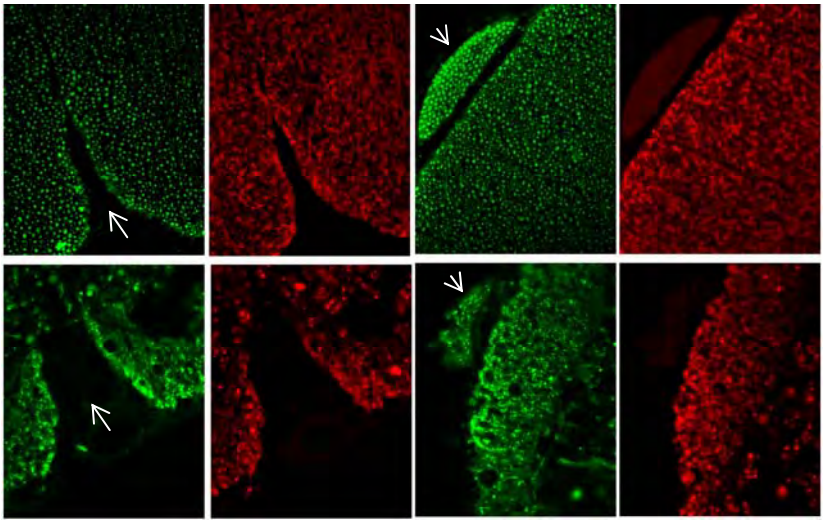
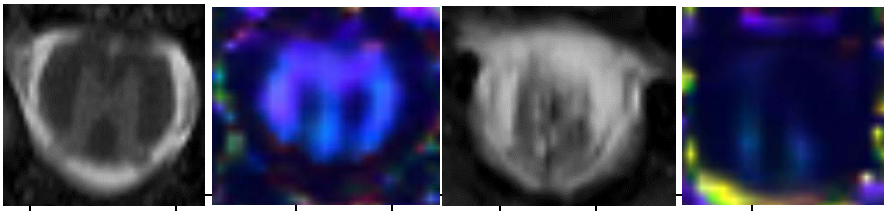


Figure 3. Axon NF200 (green) and PLP myelin (red) in uninjured (top) and SCI Rat 721 epicenter. Left ROIs are the ventral medial white matter showing the ventral median fissure (arrow). Right ROIs show lateral white matter with PLP (CNS type) myelin and also adjacent ventral roots (arrow)whose axons are myelinated with PLP-negative PNS type myelin axon (seen when sections are stained for NF200 and P0).

We have now quantified the fraction of spinal cord tissue that is immunoreactive for NF200 in different spinal cord RO1s from the 4 uninjured and the 10 selected SCI rats. The results confirm that not only is the area of the cord that contains myelinated white matter reduced in the SCI rats but the number of axons in this "spared white matter" is also significantly lower. For example, in ventral medial white matter of the 4 uninjured controls the area of tissue immunoreactive for NF200 averaged 28.5% +/- 3% while in the injured cords in was no higher than 7%. However, while controls were relatively similar in NF200 immunoreactivity for comparable ROI, the SCI rats showed considerable variability.

In order to determine if we can detect this variability with DTI imaging we are currently comparing the fractional anisotropy (FA) measures for similar regions of interest from DTI images we have obtained from these uninjured and SCI rat spinal cords, as shown in Figure 4.



DTI FA	Spinal cord	DLL WM	DLR WM	VLL WM	VLR WM
Uninj. 4	0.71	0.77	0.85	0.78	0.87
SCI 731	0.23	0.37	0.40	0.21	0.20

Figure 4. Comparison of DTI FA measures from an uninjured and a SCI spinal cord. Top panel, anatomical MRI and DTI image of Uninjured 2 rat (left) and SCI rat 731 (right) at a section through the epicenter. Note that the DTI image is more useful in detecting remaining white matter in the dorsal lateral cord (DLL, DLR) and that there is more than in the ventral lateral ROI (VLL, VLR) in this SCI.

We are continuing to analyze the DTI images that we have collected and comparing the DTI measures between control and injured rats. We will also compare the DTI results with those obtained from immunohistochemistry for axons and myelin in tissue sections from the same spinal cords. We believe the results will be useful in the future as we and others attempt to translate therapeutic results from rodents to larger mammals and from experimental to clinical settings in which tissue histopathology and immunohistochemical analyses are not possible. We expect to complete this Aim by the end of 2013.

In addition, this analysis of the histopathology of the SCI rats in Aim 2b that were not treated with GGF2 has suggested a potential explanation for not detecting any treatment effect in our efficacy study (Aim 2a). Historically, our model of SCI has created a left/right symmetrical spinal cord lesion in most rats and the sparing of white matter at the injury epicenter has been highly correlated to recovery of hind limb function as measured by the CBS and BBB. This was not true in the Aim 2 study. Of 10 untreated SCI cords that were analyzed for Aim 2b, some were symmetrical injuries as expected and shown in Figure 2. However, 6 were not and the spared white matter at the epicenter for the group as a whole did not show the expected significant correlation with 6 week functional recovery that we had seen in previous studies. Such injury asymmetry could result in plasticity from supraspinal pathways on the less injured side of the cord confounding the behavioral results and preventing us from reliably testing efficacy of the treatment.

Proposed Final Studies Based on These Results:

We will complete our investigation of the use of DTI to evaluate differences in white matter integrity after SCI, as detailed in our original approved proposal

References

Gale K, Kerasidis H, Wrathall JR. Spinal cord contusion in the rat: behavioral analysis of functional neurologic impairment. Exp Neurol. 1985 Apr;88(1):123-34.

Lytle JM, Chittajallu R, Wrathall JR, Gallo V. NG2 cell response in the CNP-EGFP mouse after contusive spinal cord injury. Glia. 2009 Feb; 57(3):270-85.

Wrathall JR, Pettegrew RK, Harvey F. 1985. Spinal cord contusion in the rat: Production of graded, reproducible, injury groups. Exp Neurol 88:108–122.

Appendix 3: SC100266 2013 Annual Report

AD_____

Award Number: W81XWH-11-1-0770

TITLE: **Translational Pharmacologic Efficacy Studies of Glial Growth Factor 2 (GGF2) in Spinal Cord Injury Models and in the Veterinary Clinical Setting**

PRINCIPAL INVESTIGATOR: Anthony O. Caggiano, PhD

CONTRACTING ORGANIZATION: CDMRP

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14. ABSTRACT

The goals of our studies in year 2 were to (a) use the most effective dosages and route of administration of GGF2 on the proliferation of NG2-expressing glia cells after SCI that we identified in year 1 in long term studies of effects on functional recovery and chronic histopathology and (b) subject spinal cords from some of these rats to MRI and Diffusion Tensor Imaging (DTI) of the spinal cord to develop MRI imaging method that can detect treatment effects that improve survival and myelination of axons after SCI for potential use in future dog studies.

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Introduction

Whittaker (2012) and Zai (2005) showed that GGF2 administration increases NG2-expressing glial progenitor cell (GPC) proliferation in surviving white matter when treatment is initiated 24 hours after spinal cord injury (SCI) in rats and mice, an effect associated with long-term beneficial effects on functional recovery following SCI (Whittaker 2012). In a recent study, subcutaneous administration of GGF2 for 1 week after SCI beginning 24 h after injury has been shown to increase the percentage of NG2-positive GPC's in residual ventral medial white matter in rats (Annual Report 22 Oct 2011 to 21 Oct 2012 for SC100266P1). Since these studies showed that GGF2 increases the number of GPC's in surviving white matter, we evaluated the effects of GGF2 using the same dosing regimen and route as well as a twice weekly maintenance dose on locomotor and bladder function recovery as well as temporal volume changes of hind limb skeletal muscle in rats following compression injury of the thoracic spinal cord. A follow-up study with a similar design with and without twice weekly maintenance dosing was recently completed.

Methods

Study 1

Eighty female Long Evans rats weighing (200-250 g) were enrolled in this study. Animals were pair housed in species-appropriate cages with enrichment items. Animals were assigned to various surgical groups based on their body weights to minimize group variance and achieve similar group body weight means. Rats were subjected to surgically-induced forceps compression-induced SCI at two severity levels (cord compressed to 0.9 mm with a rostral-caudal extent of 2.5 [moderate injury] or 4 mm [severe injury]) at the level of T9-T10 under isoflurane anesthesia. A separate group was subjected to sham-surgery.

Treatment groups:

Treatment groups were randomly assigned as follows:

Group	Animal No.	Surgical Procedure	Treatment	Dose Level (mg/kg)	Route	Regimen
1	12	0.9 X 4 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
2	11	0.9 X 4 mm	GGF2	0.24	SC	Daily for 7 days and twice weekly for 7 weeks
3	12	0.9 X 4 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
4	10	0.9 X 2.5 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
5	12	0.9 X 2.5 mm	GGF2	0.24	SC	Daily for 7 days and twice weekly for 7 weeks
6	12	0.9 X 2.5 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
7	8	Laminectomy	NA	NA	NA	NA

Endpoints:

The effects of GGF2 on open-field locomotion bladder function and changes in skeletal muscle volume were evaluated. Animals were gently handled to adapt them to behavioral testing conditions prior to surgery. Locomotor function was assessed using the 21-point open-field locomotion score (BBB) developed by Basso, Beattie and Bresnahan (Basso et al., 1995) at 2, 7 and 10 days post SCI and then weekly for 8 weeks. Urinary bladder function was evaluated daily by measuring residual urine volume (RUV) during manual bladder expression throughout the study.

Skeletal muscle volume was quantified using 3-dimensional ultrasonographic imaging of the gastrocnemius and soleus muscles on the right side at 0, 4 and 8 weeks post SCI (Vevo 2100 High Resolution Ultrasound System (Visualsonics, Toronto). Stacked images were analyzed for gastrocnemius and soleus dimensions using segmental analysis algorithms (Visualsonics, Toronto).

At the conclusion of the study, animals were euthanized under deep anesthesia by transcardial perfusion of phosphate-buffered saline followed by 4% paraformaldehyde. Spinal cords segments (area of injury and lumbar enlargement) and the medial aspect of the right lateral gastrocnemius were harvested and placed in 4% paraformaldehyde. Twenty-four hours later, muscle was transferred to 70% ethanol and the spinal cord segments were transferred to a sodium azide solution. Spinal cord segments will be subsequently paraffin embedded and sectioned using a cryomicrotome. Spinal cord tissue sections will be stained for myelin with Eriochrome cyanine in order to assess white matter sparing. Separate spinal cord sections will be evaluated for markers of neuronal plasticity (GAP43, synaptophysin and other markers). Skeletal muscle sections were stained with hematoxylin/eosin and trichrome to evaluate both the degree of skeletal musclefibrosis (degree of collagen staining) as well as myofiber size (area) distribution.

All endpoints were assessed in a manner blinded to treatment.

Mean BBB scores, RUVs, and skeletal muscle volume changes between various groups at each of the severity levels were compared using analysis of variance (ANOVA) as well as repeated measures analysis of variance over the course of the study (alpha = 0.05). When ANOVA values were significant ($p<0.05$), group means from the treatment groups were compared to the respective vehicle control as well as other treatment groups using Dunnett's and Tukey post hoc tests, respectively (alpha = 0.05).

Study 2

The methods in Study 2 were identical to those in Study 1 for surgical procedures and evaluation of the various endpoints. The difference in the study design is highlighted in the table below.

Treatment groups:

Treatment groups were randomly assigned as follows:

Group	Animal No.	Surgical Procedure	Treatment	Dose Level (mg/kg)	Route	Regimen
1	12	0.9 X 4 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
2	11	0.9 X 4 mm	GGF2	0.8	SC	Daily for 7 days
3	12	0.9 X 4 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
4	10	0.9 X 2.5 mm	Vehicle	0	SC	Daily for 7 days and twice weekly for 7 weeks
5	12	0.9 X 2.5 mm	GGF2	0.8	SC	Daily for 7 days
6	12	0.9 X 2.5 mm	GGF2	0.8	SC	Daily for 7 days and twice weekly for 7 weeks
7	8	Laminectomy	NA	NA	NA	NA

Key Research Accomplishments

Study 1

Results from Study 1 demonstrated that subcutaneous administration of GGF2 24 h post-injury produced significant improvements in locomotor function as assessed by the BBB locomotor rating scale following severe and moderate compression SCI (see Figures 1 A and B, respectively).

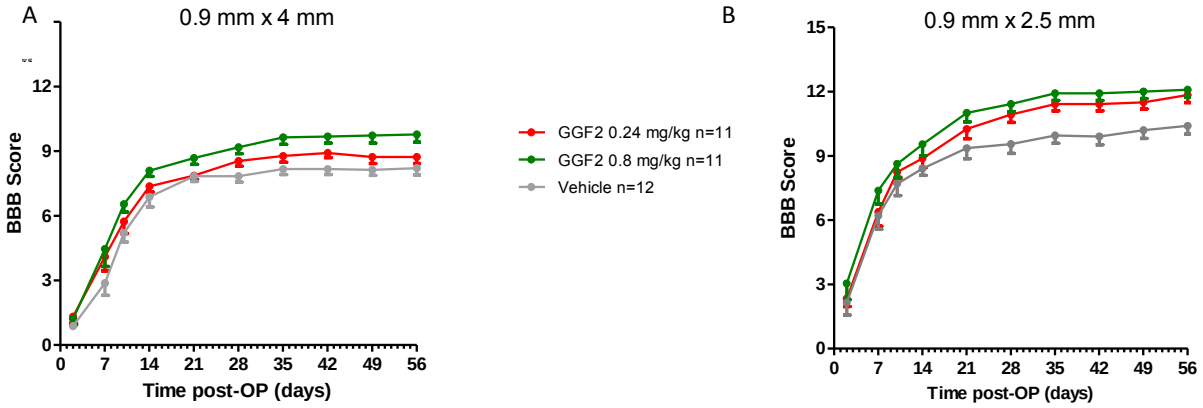

Figure 1. Effects of GGF2 on overground locomotion following severe (A) and moderate (B) compression SCI. Data are presented as mean ± SEM. A and B. $p<0.05$ GGF2 at both doses vs vehicle (repeated measures ANOVA).

Locomotor function was not only statistically improved by GGF2, but the changes observed in open-field locomotion were considered biologically meaningful in that at both severities of injury, the GGF2 treatment led to functionally relevant improvements on the BBB scale. Specifically, GGF2-treatment improved coordination of hind limbs during locomotion compared to vehicle treatment in moderate SCI. In severe SCI, GGF2 promoted plantar stepping compared to vehicle treated animals. Further, GGF2 treatment also improved bladder function as assessed by residual urine volumes (RUVs, Figure 2). In this case, significant reductions in residual urine volumes suggesting improvements in spontaneous voiding over time were observed at both injury severities with GGF2 treatment relative to vehicle (both dose levels in the case of moderate injury, high dose only in the case of severe injury). Skeletal muscle volume did not change significantly post-SCI for either the gastrocnemius or soleus muscles over time compared to laminectomy control animals (moderate injury subjects analyzed only, data not shown). Although GGF2 appeared to improve soleus muscle volume at a dose of 0.8 mg/kg compared to vehicle controls in the moderate injury cohorts, the effects on skeletal muscle volumes were not statistically significant at any dose tested (Figure 3). Muscle volume changes from the severe injury group are being analyzed.

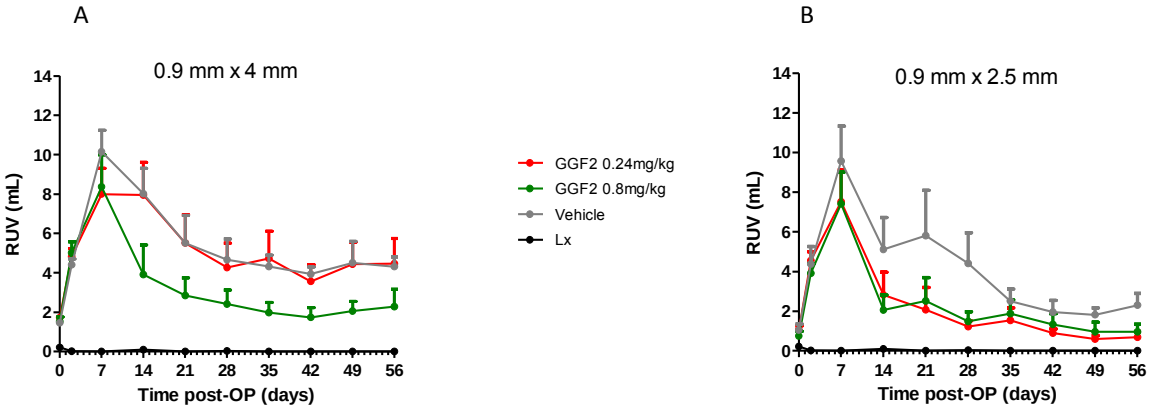

Figure 2. Effects of GGF2 on bladder function (RUVs) following severe (A) and moderate (B) SCI. Data are presented as mean ± SEM. A. $p<0.05$ GGF2 0.8 mg/mL vs vehicle. B. $p<0.05$ GGF2 at both doses vs vehicle (repeated measures ANOVA).

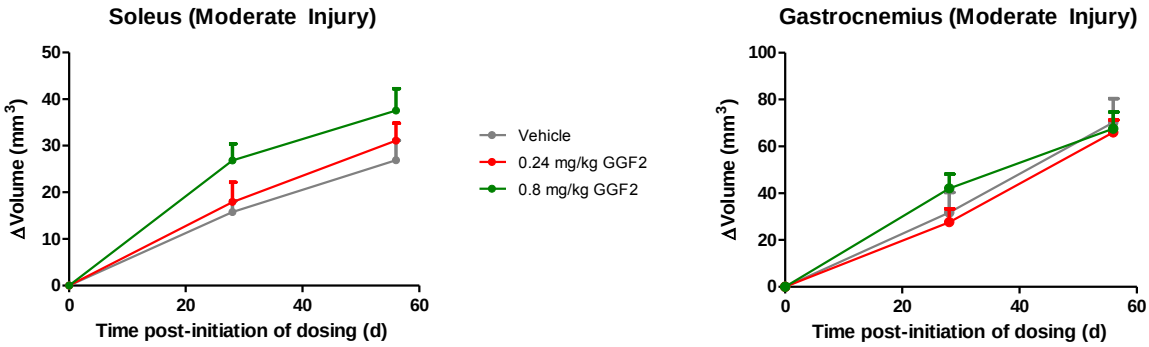

Figure 3. Effects of GGF2 on changes in gastrocnemius and soleus muscle volume (mm^3) over time following moderate SCI. Data are presented as mean ± SEM.

Study 2

Results from Study 2 demonstrated that subcutaneous administration of GGF2 24 h post-injury produced only modest improvements in locomotor function as assessed by the BBB locomotor rating scale following severe and moderate (see Figures 5 a and b, respectively) SCI. The magnitude of the effect observed in the daily dosing plus maintenance arm of this study was substantially less than that observed in the corresponding arm of maintenance Study 1. The difference in BBB scores in the moderate injury group with maintenance dosing in Study 2 was not sustained at the last two observation time points.

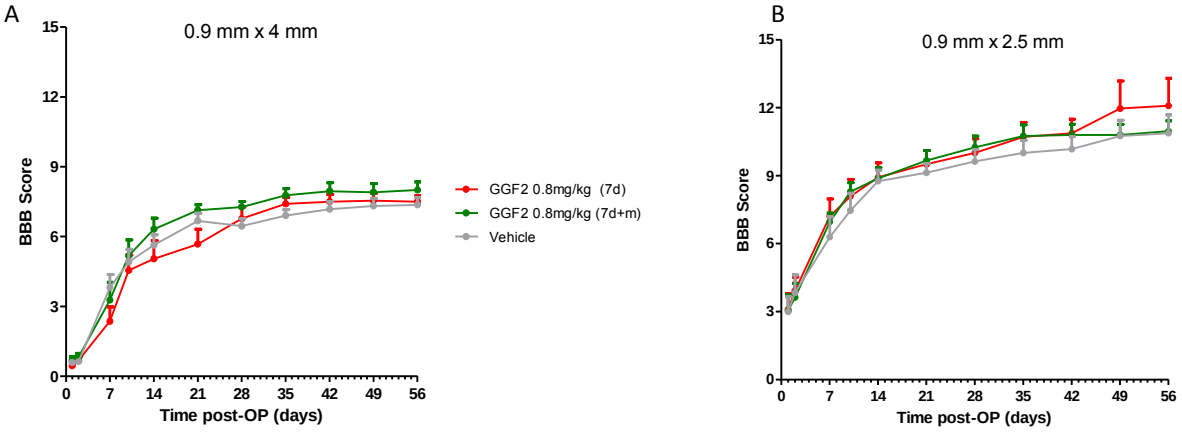

Figure 5. Effects of GGF2 on overground locomotion following severe (A) and moderate (B) SCI. Data are presented as mean ± SEM. . A. $p<0.05$ GGF2 0.8 mg/kg with maintenance dosing vs vehicle. B. $p<0.05$ GGF2 both dosing regimens vs vehicle (repeated measures ANOVA).

GGF2 treatment had no significant effect on RUV with either dosing regimen in the severe injury group but significantly improved RUV in the 7 day treatment group without maintenance regimen compared to vehicle treatment in the moderate injury group (see Figure 6 A and B). Gastrocnemius and soleus muscle volumes are not reported since image analysis is ongoing.

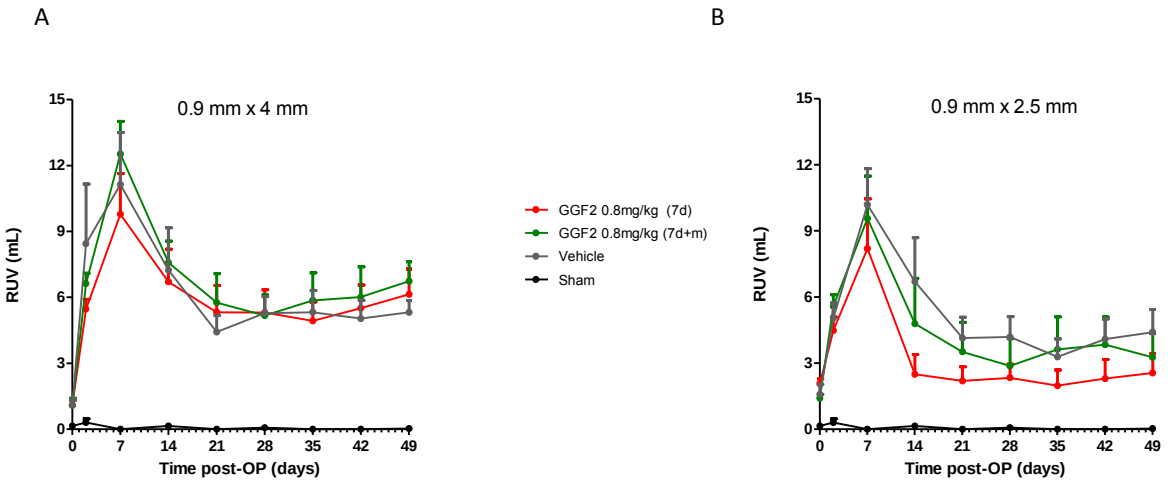

Figure 6. Effects of GGF2 on bladder function (RUVs) following severe (A) and moderate (B) SCI. Data are presented as mean ± SEM. A. No significant differences. B. $p<0.05$ GGF2 0.8 mg/kg (repeated measures ANOVA).

Reportable Outcomes

Animal models of SCI should bear clinical relevance for understanding disease pathophysiologic changes and developing therapies. SCI not only impacts locomotor function, but also produces skeletal muscle atrophy and autonomic dysfunction at and distal to the level of injury. These pathophysiologic elements are reproduced to a certain extent in the forceps compression model of rat spinal cord contusion injury used in these studies. In addition to capturing the features of human spinal cord injury, the forceps-compression model is also sensitive to drug treatment. Specifically, Caggiano and coworkers (1995) showed that a bacterial enzyme improved locomotor and bladder function in this model. The present studies employed this model to evaluate the effects GGF2 on locomotor and bladder function as well as skeletal muscle volume following SCI.

Locomotor function

Subcutaneous administration of GGF2 24 h post-injury produced improvements in locomotor function as assessed by the BBB locomotor rating scale following severe and moderate SCI. In Study 1, locomotor function was not only statistically improved by GGF2 vs vehicle treatment, but the changes observed in open-field locomotion were biologically meaningful (e.g. improved coordination and promoted plantar stepping in moderate and severe injury, respectively) in that at both severities of injury the GGF2 treatment led to functionally relevant endpoints on the BBB scale. In contrast, only modest improvements in locomotor function were observed with GGF2 treatment compared to vehicle controls in Study 2. While the some of the effects in Study 2 were statistically significant, the changes observed in

recovery of locomotion following GGF2 treatment were deemed to be consistent but modest and in some cases not to be functionally relevant or not sustained by the final time point .

Bladder function

SCI above sacral levels leads to significant autonomic dysfunction as evidenced by an impaired micturition in humans and in rats following SCI. In rats, RUV is used to assess urinary bladder function (Caggiano, 2005; Mure, 2004; Kruse 1993). The results from Study 1 suggest that GGF2 produces a significant reduction in RUV, implying improved efficiency in bladder emptying. In Study 2, the effects of GGF2 on bladder function were less pronounced than in Study 1.

Skeletal muscle changes

The reduction in skeletal muscle mass typically observed following spinal cord injury is related to a reduction in both type I and II skeletal muscle fiber size and occurs rapidly (within 10 days) in humans (Dupont-Versteegden, 1998). It is important to preserve as much skeletal muscle as possible in order to maintain muscle strength for rehabilitation and avoid metabolic syndromes associated with muscle loss. Although SCI typically produces skeletal muscle atrophy at levels below the injury, no significant reductions in target hind limb muscle volumes following SCI were noted in Study 1 (moderate injury). Analysis is ongoing for the severe injury group in Study 1 and all groups in Study 2.

Conclusion

In conclusion, the results presented here provide evidence that GGF2 improves locomotor function following moderate and severe compression injury when treatment is initiated 24 hours after forceps compression spinal cord injury. Although improvements in locomotor function produced by GGF2 in both studies were statistically significant, the magnitude of the effects on locomotion varied greatly from study to study ranging from what is considered biologically meaningful in study 1 to much smaller changes in study 2. Study 1 provided preliminary evidence that GGF2 treatment improves bladder function following thoracic SCI; however, bladder function in Study 2 was not improved. Although the effects of GGF2 treatment on skeletal muscle changes were not significant (Study 1), histological analysis is ongoing to determine the effects of GGF2 on microscopic changes in the muscle. Additional work is required to determine the reproducibility of these findings, the mechanisms of GGF2 action, optimal treatment regimens and potential side effects.

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Appendix 4: SC100266 Wrathall Year 3 Progress report

AWARD NUMBER: W81XWH-11-1-0771

TITLE: Translational Pharmacologic Efficacy Studies of Glial Growth Factor 2 (GGF2) in Spinal Cord Injury Models and in the Veterinary Clinical Setting

PRINCIPAL INVESTIGATOR: Jean R. Wrathall, PhD

CONTRACTING ORGANIZATION: Georgetown University, Washington, D.C.

REPORT DATE: December 26, 2014

TYPE OF REPORT: Annual

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

In year 3 of this collaborative project to determine if GGF2 could be efficacious in treating spinal cord injury (SCI) in experimental rat models then in natural dog SCI we (3.1) compared histopathological data from SCI rat tissue from the Georgetown and Acorda models of incomplete thoracic injury to see similarities and differences in these rodent SCI models we used to examine GGF2 treatment effects and (3.2) compared the histopathological and immunohistochemical data from the Georgetown model with result from analyses of the same spinal cords by MRI and DTI to determine if we could develop a useful method for using DTI to examine histopathology in a non-invasive way to support further studies in large animals (such as dogs) and in humans with SCI .

15. SUBJECT TERMS

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1. **INTRODUCTION:** In order to test the hypothesis that treatment with Glial Growth Factor 2 (GGF2) administered after spinal cord injury could be translated into an effective treatment to reduce the effects of spinal cord injury (SCI) we proposed in Aim 1 to determine the optimal dose and route of administration of GGF2 in terms of its effects on stimulating proliferation of NG2-expressing glial progenitor cells using an established rat model of incomplete contusion SCI. We completed Aim 1 in year 1 of the project. Aim 2A was to test the long term effects of this dose and route in terms of functional recovery from SCI using the rat model of weight drop SCI that we use at Georgetown. We completed Aim 2A in year 2 of the project and began our final Aim, 2B, to use MRI and DTI analysis of injured rat spinal cords and determine the extent to which the data could serve in lieu of detailed histopathological and immunohistochemical analysis of the tissue that would not be possible with large animal models (e.g., dogs) or patients with SCI. In year 3 of the project we (3.1) compared histopathological and immunohistochemical data from SCI rat tissue from the Georgetown and Acorda models of incomplete thoracic injury and (3.2) compared the behavioral, histopathological and immunohistochemical data from the Georgetown model with result from analyses of the same spinal cords by MRI and DTI and investigated methods of analyzing the DTI data that would provide optimal information on actual histopathology.

2. **KEYWORDS:** spinal cord injury, GGF2, neuregulin, MRI, DTI,

3. **OVERALL PROJECT SUMMARY:** In year 3 of the project we (3.1) compared histopathological and immunohistochemical results from SCI rat tissue from the Georgetown and Acorda models of incomplete thoracic injury and (3.2) compared the behavioral, histopathological and immunohistochemical data from the Georgetown model with results from different approaches to analysis of the same spinal cords by MRI and DTI.

3.1 To assist interpretation of the joint results from the Georgetown and Acorda investigations of the effect of GGF2 on rat models of SCI, I examined and compared the lesion sites from rats subjected to the forceps compression model of SCI employed at Acorda with that from the weight-drop contusion model of injury used at Georgetown.

My analysis of Acorda tissue was based on histological sections through the injury sites that they had prepared commercially from paraffin embedded tissue from a subset of the spinal cords of rats that were representative of the larger control and experimental groups generated at Acorda. The tissue cross sections from near the center of the SCI site were stained with luxol blue (for myelin) and counterstained with H&E (for cells and nuclei) and sent to us with only sample number designations. Figure 1 shows low magnification images I took of sections from four of these rats (numbers 70, 73, 76 and 79).

I also examined the stained sections at higher magnifications to compare cellular characteristics of the lesions and surrounding spared tissue. I found many similarities and a few differences from that typical with the weight drop model of rat SCI used at Georgetown. The results and additional images to document them were conveyed to Acorda

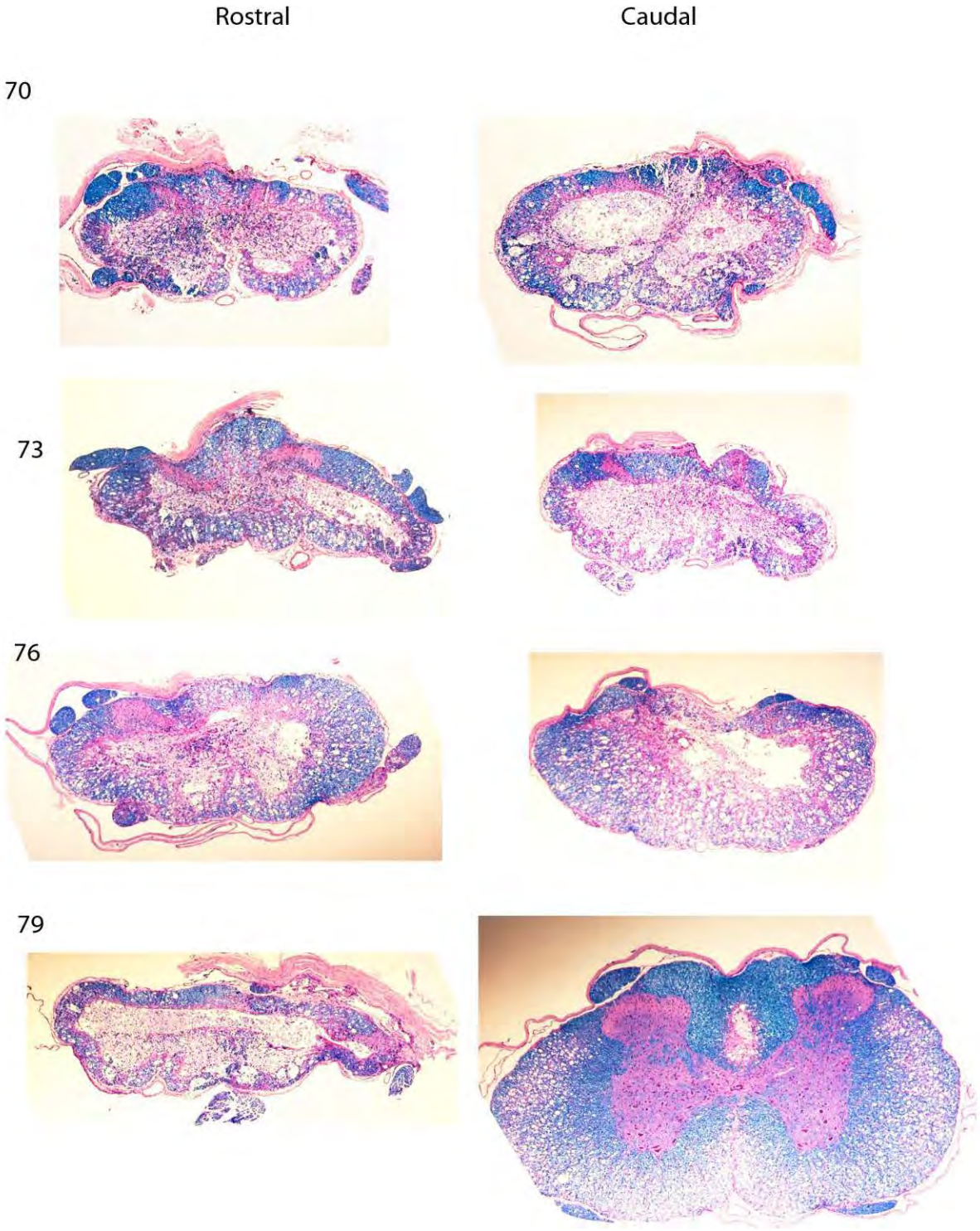

Figure 1. Coronal (cross) sections of the spinal cord injury sites from 4 rats (numbers 70, 73, 76 and 79) subjected to forceps compression SCI at the Acorda laboratory. Residual myelin is stained with luxol fast blue (LFB) and non-myelinated areas of spared gray matter as well as lesion tissue are stained pink and purple with H&E.

3.2 To complete Aim 2B of the approved Georgetown project I collaborated with the Preclinical Imaging Research Laboratory (PIRL) of the Lombardi Cancer Center at Georgetown University directed by Dr. Chris Albanese and with Dr. Olga Ramirez of that laboratory in detailed MRI DTI and immunohistochemical image analyses of tissue sections through injury epicenters from injured but untreated (injured control) rats from our Aim 2A studies.

Ten injured control SCI rats were selected based on their BBB hindlimb locomotor scores (BBB = 21 in a normal rat) at 6 weeks after SCI to represent severe (n = 3, BBB = 8-9), moderate (n= 4, BBB 12-13) and mild (n = 16-19) injury in order to have the greatest chance of significant differences in tissue sparing and other aspects of histopathology that might be detectable by DTI analysis. Five additional rats were used as uninjured controls and analyzed in parallel to the SCI rats.

Rats were anesthetized and transcardially perfused with phosphate buffered saline (PBS) followed by 4% buffered paraformaldehyde. Spinal columns were removed and stored in 10% sucrose for MRI imaging at the PIRL. The MR imager used is a 7.0 Tesla horizontal Bruker magnet with a 20 cm bore equipped with 100 gauss/cm microimaging gradients and run by Paravision 5.0 software. The samples were imaged in a 40 mm transmit-receive volume RF coil. To acquire anatomical images, a two-dimensional T2–weighted Turbo RARE (rapid acquisition with rapid enhancement) protocol was used with 30 interlaced slices in the axial plane, TR: 4235 ms, TE: 36 ms, RARE factor: 6, averages: 4, field of view: 25 X 25 mm, matrix: 256 x 256, and slice thickness of 1 mm. Subsequently, two-dimensional DTI was performed with TR: 12500 ms, TE: 21.1ms, FA: 90 deg, eff BW: 300000 Hz, segments: 4, averages: 8, diffusion directions: 30, b: 1900s/mm2, slice thickness: 1mm, FOV: 25 mm x 25 mmm. Fractional anisotropy of white and gray matter in the ex-vixo thoracic spinal cord of the injured versus uninjured subjects was measured in selected regions of interest (ROI) ranging from the entire area of the cord to selected smaller areas that normally contain white matter.

After MRI the spinal cords were removed from the spinal columns and serial 20μm frozen sections were prepared through a 1.5 cm length segment of cord centered on the T8 injury site. Sections of tissue representing each mm of cord segment were stained with Eriochrome to color myelin blue and counterstained with H&E to show spared gray matter and lesion cells. Examination of these sections allowed identification of the injury epicenter as the location with the least residual white matter. Adjacent slides were identified to represent the epicenter and specified distances rostral and caudal to it. In addition, the area of spared white matter was calculated from erichrome stained sections of the epicenters and sections up to 4 mm rostral and caudal to it.

Immunohistochemistry was performed on spinal cord cryostat sections from MRI-imaged tissue at the injury epicenter and 1 mm rostral and caudal to the injury epicenter using antibodies to axons (NF200) and both central (PLP) and peripheral (PO) myelin. Fluorescent secondary antibodies were used to detect immunostaining and images were obtained from a Zeiss fluorescent microscope. Analysis of fractional percent of tissue that was immunostained in specified regions of interest was performed using NIH Image J.

A number of different methods to compare DTI results with qualitative and quantitative measures of histopathology as well as functional impairment and overall white matter sparing were examined. In summary, we found that at the injury epicenter the overall (using the entire spinal cord cross section as the ROI) fractional anisotropy (FA mean) as well as the FA ID (corrected for the area of the injured spinal cord) was significantly lower in each of the injured spinal cords as compared to the uninjured controlled. The table below shows examples of these data.

Rat #	BBB	FA mean	FA ID
Uninjured 1	21	0.63	90
721	8	0.23	9
725	8	0.25	17
705	12	0.31	20
727	13	0.20	20
709	19	0.24	18

However, as shown in the table, the differences in these two measures among SCI rats did not seem to be related to each other or to distinguish between rats with differences in functional recovery (BBB) or histopathology of the injury sites (data not shown)

We also compared differences in the component of FA that results from longitudinally directed structures such as axons in spinal white matter (First EV). As shown in Figure 2, although all of the SCI cords had a much reduced density compared to uninjured controls (group on upper right) this measure did not distinguish between mild, moderately and severely injured subjects) cords, or measures of white matter of neurofilaments density (data not shown. .

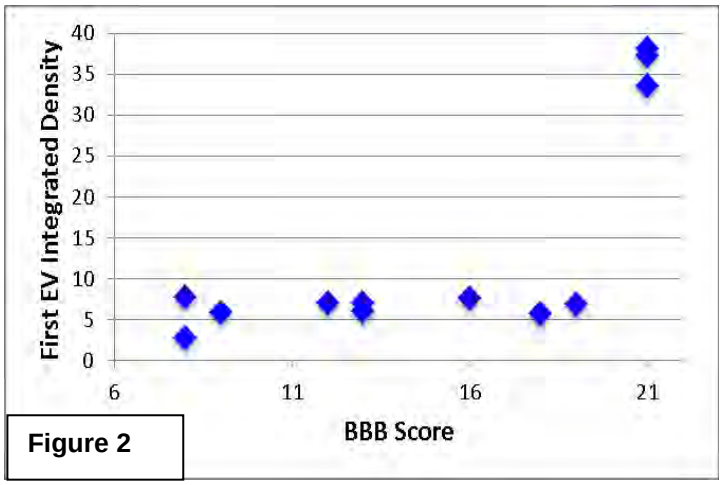

Figure 2

However, a consistent measurement of white matter and axon integrity could be detected by specific region of interest (ROI) measures in the dorsal (D), left a right dorso- and ventolateral (DLL, VLL, VLR, DLR) and ventral (VM) white matter of uninjured control spinal cords as shown on the right, from which a plot of the normal peripheral rim of white matter can be visualized, as shown in Figure 3, on the next page

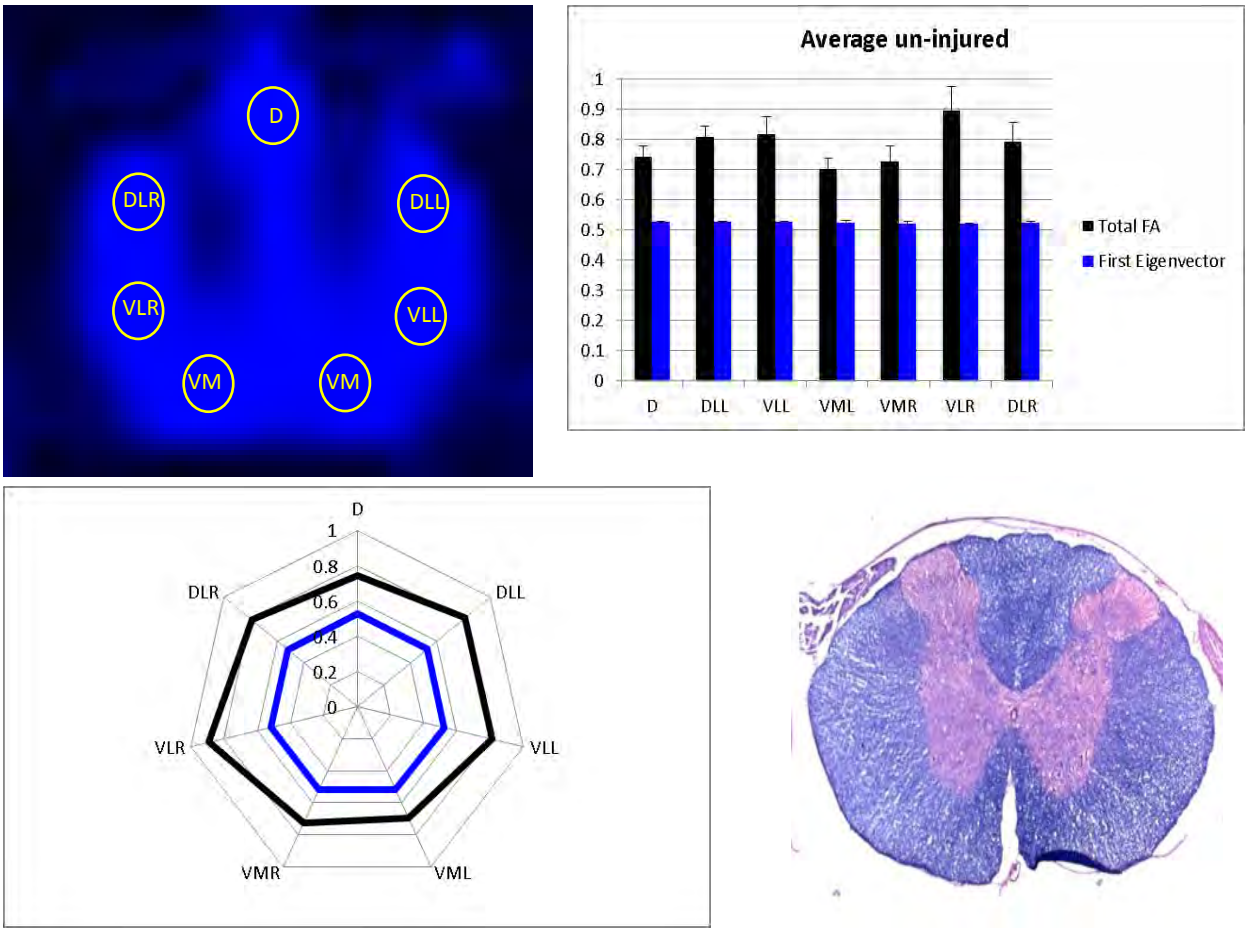

Figure 3. White matter ROI analysis of Total FA (black) and the longitudinal component of FA (blue) for mid thoracic spinal cords of uninjured control rats (n = 4) subjected to MRI and DTI with analysis of the specified dorsal, lateral and ventral ROI locations (upper left). The mean and SEM of values at each of the ROI locations show that longitudinal FA (first Eigenvector) is especially consistent in the uninjured spinal cords (upper right). Radial plots of average values for the uninjured cords (lower left) show a pattern consistent with the typical histology in Eriochrome (blue, white matter) and H&E (pink/purple, gray matter) stained histological sections of uninjured thoracic cord (lower right) showing well-myelinated peripherally located white matter in the dorsal, lateral and ventral locations analyzed..

We are currently completing analysis of all the injured spinal cords using this method to test the hypothesis that using this new ROI approach can detect important differences in the histpathological nature of the injury in different subjects.

4. KEY RESEARCH ACCOMPLISHMENTS: Nothing to report.

5. CONCLUSION: Our new DTI analysis procedure holds promise as a potential non-invasive method to detect important histopathological parameters of SCI in large animal models and humans with SCI using non-invasive MRI DTI. Our plan is to complete these analyses of the rat data as soon as possible.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS: Nothing to report.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS: Nothing to report.

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11. APPENDICES: NA

Appendix 5: SC100266 Olby Final report

AD_____

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TITLE: Translational Pharmacologic Efficacy Studies of Glial Growth Factor 2 (GGF2) in Spinal Cord Injury Models and in the Veterinary Clinical Setting

PRINCIPAL INVESTIGATOR: Dr. Natasha Olby
Note this is a multi-institutional grant with Dr. Anthony Caggiano of Acorda Therapeutics serving as the initiating PI and with Dr. Jean Wrathall serving as PI at Georgetown Medical Center.

CONTRACTING ORGANIZATION:
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13. SUPPLEMENTARY NOTES

14. ABSTRACT The purpose of this translational project is to identify the most appropriate dosing schedule and administration route of glial growth factor 2 (GGF2) in the treatment of rodent spinal cord injury (SCI) and to translate this work to dogs with naturally occurring (SCI). In our last report we summarized hypersensitivity issues we encountered; a revised statement of work was approved to further explore the tolerability of GGF2 in dogs with and without SCI. GGF2 was administered SC (0.08mg/kg) to 10 dogs once a day for 7 days; 5 dogs had intrathecal catheters placed for 6 hours to mimic previous experimental conditions. An equal volume of vehicle was administered in the same way to 5 additional dogs. Adverse effects were not detected in any of the dogs during administration or the following 3 months. Based on this, 10 dogs with naturally occurring spinal cord injuries due to disc herniations were recruited to a phase 1 safety trial. Dogs entering the study were paraplegic with no pain perception and presented within 24 hours of onset of paralysis. All underwent advanced imaging to identify their herniated disc and were surgically decompressed. Dogs were randomized to receive GGF2 or vehicle at a dose rate (or equivalent volume) of 0.08mg/kg SC once a day for 7 days. Treatment was initiated as soon as the diagnosis was confirmed. Presence of adverse effects and recovery were monitored daily during the 7 days of postoperative hospitalization, and then at 2,4 and 8 weeks postoperatively, when an MRI was repeated. There were no adverse effects noted in any of the dogs during GGF2 administration. dogs receiving GGF2 and dogs receiving vehicle recovered independent ambulation, one dog receiving placebo was euthanized due to ascending myelomalacia, and the remaining dogs remained paraplegic without pain perception. We conclude a single course of 7 days of once daily SC GGF2 is safe in dogs with acute spinal cord injury. Efficacy has not yet been evaluated.

15. SUBJECT TERMS Spinal cord injury; GGF2; tolerability, dog

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Introduction

The objectives of this multi-institutional translational project are to determine the most appropriate route and dosing regimen for GGF2 in the treatment of acute spinal cord injury in rodents, to test the tolerability and the pharmacokinetics of GGF2 in dogs using the data established in the first phase of the work and then to test this dosing regimen for efficacy in a naturally occurring model of canine spinal cord injury. In Project 3, aim 4, the tolerability and pharmacokinetics of GGF2 were evaluated in healthy dogs and hypersensitivity issues were encountered. The half-life of GGF2 was established in dogs following intravenous and SC administration. As a result, a changed plan of work was submitted to confirm the safety of a single course of GGF2 in healthy dogs at the dose established in the previous work and then to move on to a phase 1 safety study in dogs with naturally occurring spinal cord injury. In aim 4b we confirmed that a one-week course of SC GGF2 at a dose rate of 0.08mg/kg once a day was well tolerated with no adverse side effects. In aim 5, we showed that the same dosing regime was well tolerated in dogs with acute severe spinal cord injuries due to intervertebral disc herniations. This report summarizes the results of Aim 5 and includes pharmacokinetic data from Aim 4 that has not yet been reported. The other reports are summarized and referenced for previous data.

Body

Project 3: To determine the tolerability, pharmacokinetics of GGF2 and its efficacy at improving outcomes from naturally occurring thoracolumbar SCI in dogs.

Aim 4 (months 18-24): To establish the tolerability and pharmacokinetics of GGF2 in dogs.

In the first phase of study under Aim 4, we administered GGF2 SC once a day for 7 days to 6 healthy dogs at 2 different doses shown to have efficacy in the initial phases of this work (0.24 and 0.08mg/kg: canine equivalent of 0.8 and 0.24 mg/kg in rats) and we sampled blood and CSF for pharmacokinetic analysis. We encountered minor issues with injection site pain at the higher dose (0.24mg/kg) but there was no evidence of this effect at the 0.08mg/kg dose or when vehicle alone was administered to 3 healthy dogs. While GGF2 was administered SC as determined to be effective in the initial phases of work, it was administered IV to 2 dogs on the first and last days of dosing to optimize pharmacokinetic data. These 2 dogs developed severe hypotension on the second round of dosing, likely as a result of sensitization to this human recombinant protein in the first round of drug administration. In addition, 4 of the 6 dogs developed late onset transient cerebellar signs, also believed to be a result of sensitization in the first round of dosing. *See grant report dated 24/10/2013 for full summary and data.* On the basis of these findings, a revised statement of work was approved to further explore the tolerability of GGF2 in dogs with and without spinal cord injury.

Measurement of GGF2 levels in the blood of these 6 dogs was completed by Acorda Therapeutics using a quantitative ELISA method. The full report of these results including details of the method, generation of standard curves and all data is provided in Appendix 1. In all but one dog, blood levels were not measureable following SC administration, but IV administration resulted in detectable levels and pharmacokinetic parameters were calculated. This data is provided in the table below:

	Dose_Number											
	1						2					
Dog_	t ½ h	Tmax h	Cmax ng/mL	Tlast h	AUClast h*ng/mL	AUCall h*ng/mL	t ½ h	Tmax h	Cmax ng/mL	Tlast h	AUClast h*ng/mL	AUCall h*ng/mL
1	0.32	0.083	275.36	1	89	101	0.24	0.083	312.22	1	79	88
2	0.14	0.083	156.27	1	36	41	0.29	0.083	409.31	1	107	118
3	0.16	0.083	1487.49	1	277	283		-	-	-	-	-
4	0.24	0	175.19	1	51	61						

Table 1: Pharmacokinetic parameters for 3 dogs following IV administration and 1 dog (dog 4) following SC administration of a single dose of GGF2. Dose number 1 is the first dose given. Dose number 2 is the second IV dose given after a week of daily SC doses. T1/2: half life; Tmax: time to maximum concentration; Cmax: maximum concentration: Tlast: time of last point with quantifiable blood levels; AUClast: area under the concentration time curve to the last measureable concentration; AUCall: the total area under the concentration time curve, representing the total exposure to drug.

Aim 4b (months 25 – 29): Further investigation of tolerability of GGF2 in dogs:

In aim 4a we established that a dose of 0.24mg/kg administered SC once a day for 7 days was tolerated well systemically but produced mild injection site pain. Injection site pain was not seen with a dose of 0.08mg/kg. However, a hypersensitivity reaction (anaphylaxis) was encountered in the second round of 7 days of GGF2 dosing associated with IV administration. In addition, 4 of 6 dogs developed transient cerebellar signs in the first 3 weeks after the second round of dosing that we postulated may reflect CNS hypersensitivity secondary to repeated SC administration of GGF2 and placement of intrathecal catheters (causing CNS inflammation). In order to establish the safety of administering GGF2 SC once daily for 7 days only (no additional dosing beyond one week of dosing), additional testing in 3 groups of 5 beagles was undertaken.

Dogs in groups 1 and 2 received GGF2 at a dose of 0.08 mg/kg SC q24h for 7 days. Group 2 dogs had an intrathecal catheter placed for 6 hours via the lumbar cistern and then removed on day 1 to incite CNS inflammation (mimicking the experimental conditions of Aim 4a and producing some inflammation, as would be encountered with spinal cord injury). The technique used was identical to that described in the previous report. Briefly, following induction of general anesthesia, lumbar puncture was performed with a 2.5 inch 20G spinal needle and a 24G epidural catheter (ReCath Co) was placed intrathecally via this needle. Flow of CSF was confirmed prior to removal of the spinal needle, and then the catheter was capped of the catheter and anchored to the skin using the supplied foam pad and sutures. The external catheter was covered with a sterile dressing and the dogs were recovered from anesthesia. Intrathecal catheters were placed without problem in all dogs, and none of the dogs interfered with them. They were removed uneventfully after 6 hours. Group 3 received an equal volume of vehicle SC q24h for 7 days. Serum chemistry panels and complete blood cell

counts were performed prior to the first dose and then at 3, 7, 21 and 90 days. All dogs were monitored for 3 months in total to ensure that there were no late developing side effects.

None of the dogs in this follow up study developed any adverse effects during GGF2 administration or in the subsequent 3 months of daily monitoring. There were no significant changes in CBC or serum chemistry panels (*data submitted in report dated October 2014*). Based on the apparent safety of a single 7-day course of GGF2 administered at a dose rate of 0.08mg/kg SC once a day, we moved on to complete Aim 5.

Aim 5 (months 30-36): To establish the safety of GGF2 in dogs with naturally occurring, severe spinal cord injuries.

Ten dogs with naturally occurring acute intervertebral disc herniations causing clinically complete thoracolumbar spinal cord injuries were recruited from dogs presenting to the neurology service at NCSU College of Veterinary Medicine. Details of the study were explained to the owners, including the results of the initial tolerability testing, and owners who wished to take part signed a consent form. As is standard for severe acute disc herniations, diagnostic testing and treatment was initiated immediately on an emergency basis. All dogs underwent a standard diagnostic workup including a complete blood cell count (CBC) and serum chemistry panel, radiographs of the spine, followed by computed tomography (CT) or magnetic resonance imaging (MRI) of the spine and immediate surgical decompression of the herniated disc material. Dogs were randomized to vehicle or GGF2 prior to starting the study and the first dose was administered as soon as the diagnosis of a disc herniation was confirmed with imaging. Injection sites were rotated around the trunk and were monitored daily for signs of pain or inflammation.

Outcome measures included an open field score (OFS), stepping and coordination treadmill scores to assess gait, a dichotomous assessment of being able to walk independently (yes or no), time to urination, percentage bladder emptying, and assessment of pain perception and proprioceptive placing. Gait assessments were achieved by videotaping the dog walking on a treadmill at a comfortable speed, with tail support if needed, and on a nonslip mat (for the OFS). Details of the development and validation of these scoring techniques are published elsewhere[1,2]. Pain perception and proprioceptive placing were scored with ordinal scales (Table 2) and the presence or absence of pain perception was also scored (yes or no). Pain perception was assessed using forceps to clamp on the medial and lateral toes of the hind feet, applying gentle pressure until the dog acknowledged the stimulus. Instrumented forceps were used that allowed assessment of the pressure applied. Proprioception was assessed by supporting the dogs' hindquarters with a hand under their abdomen, and placing each foot onto its dorsal surface. A normal response is a rapid replacement of the foot into a normal position.

Parameter	Scoring per hind limb	Total normal score
Proprioceptive placing	0:absent 1:reduced 2:normal	4
Pain perception - Hind limbs - Tail	0:absent 1:reduced 2:normal	6

Table 2: Ordinal scales used to score pain perception and proprioceptive placing.

Urination was assessed by observation of when the dogs started to urinate voluntarily, and ultrasound of their bladders to measure the maximum length and depth in 2 planes, longitudinal and transverse, before and after urination and calculating the volume and percentage emptying from these values as described elsewhere[3]. All measures were assessed daily from twenty-four hours after surgery until discharge at one week and then again at 2, 4 and 8 weeks after surgery. At 8 weeks, a spinal MRI was performed as well as assessment of spinal cord conduction with spinal evoked potentials. A CBC and serum chemistry panel was repeated on day 3, 7 and at the 8-week study endpoint.

Ten dogs were recruited to the study. Details of their breed, age, sex, diagnostic work up, site of disc herniation, and surgical treatment are given in Table 3. All dogs were acutely (less than 24 hours) paraplegic with no pain perception on entry into the trial as per the trial inclusion criteria. All dogs tolerated their daily injections with no adverse events occurring and no unexpected changes in their blood work (*see report dated October 2014 for full data*).

Dog	GGF2 or V	Breed	Age	Sex	Preop imaging	Site of disc herniation	Surgery
1	GGF2	Dachshund	2y	F	CT	L1/2	Hemilam L1/2 on right
2	V	Poodle	7y	MC	MRI	L3/4	Hemilam L3/4 on left
3	V	Cocker spaniel	8y	FS	MRI	T12/13$	Hemilam T11-13 on left. Cord changed color intra-operatively
4	GGF2	Dachshund	4.5y	FS	CT	T12/13	Hemilam T11-13 on right.
5	GGF2	Cocker spaniel	4.5y	M	MRI	T11/12$	Hemilam T11-13 on left
6	V	Cocker spaniel	4y	FS	CT	T11/12/13	Hemilam T11-L1 on left
7	V	Dachshund	3y	FS	MRI	L3/4/5 *	Hemilam L3/4, pediculectomy L4/5
8	GGF2	Dachshund X	4.5y	FS	MRI	L3	Hemilam L2/3/4 on right
9	V	Labradoodle	6y	MC	CT	L2/3/4	Hemilam L2/3/4 on right
10	GGF2	Dachshund	4y	FS	CT	L1/2	Hemilam L1/2 on left

Table 3: Signalment and details of diagnosis and treatment of disc herniations for dogs in phase 1 trial. V: Vehicle. MC: male castrated; FS: female spayed; CT: computed tomography; MRI: magnetic resonance imaging; T: thoracic; L: lumbar; Hemilam: hemilaminectomy.$: extensive cord edema present on MRI; *: incidental syrinx present in caudal thoracic spinal cord.

The outcome data on gait, pain perception, proprioception and bladder function are presented in Table 4. Percentage bladder emptying altered over time, and so we have provided the maximum percentage emptying in the 8 week period assessed.

Dog	Walking Y/N	OFS	SS (%)	RI (%)	PP Y/N	PP score	Prop score	Days to urination	% emptying
1: GGF2	N	0	0	0	N	0	0	NA	NA[@]
4: GGF2	N	4	20.4	0	Y	2	2	NA	NA[@]
5: GGF2	Y	9	94.1	48.5	Y	6	4	8	90 (WNL)
8. GGF2	Y	8	83.7	8.9	Y	6	4	7	75.05
10. GGF2	Y	8	72	18.6	Y	6	3	14	96 (WNL)
2: V	Y	11	100	100	Y	6	4	5	95.6 (WNL)
3: V*	N	0	NA	NA	N	NA	NA	NA	NA
6: V	Y	6	66	19.2	Y	1	0	NA	NA[@]
7. V	N	0	0	0	N	0	0	NA	NA[@]
9.V	Y	10	100	64	Y	6	4	7	WNL[#]

Table 4: Outcomes for dogs in phase 1 trial. V: vehicle. OFS: open field score; PP: pain perception; Prop: proprioception; NA: not applicable; WNL: within normal limits. * Euthanized on day 2 due to development of ascending myelomalacia. [@]: voluntary urination not regained. [#]: Dog urinating normally at home, would not urinate at hospital for bladder measurements (behavioral).

This trial was a safety trial including a low number of dogs, and not intended as an efficacy trial. Overall, 2 dogs in the GGF2 group failed to recover the ability to walk independently, and 1 dog in the vehicle group was euthanized due to development of ascending myelomalacia (a fatal condition that occurs in approximately 17% of dogs with acute intervertebral disc herniations causing clinically complete lesions), and 1 dog failed to recover. Statistical comparison of the 2 groups outcomes was performed using the Kruskal-Wallis test for continuous data and by constructing contingency tables and performing a chi-square test for categorical data. $P < 0.05$ was taken as significant. There were no significant differences between groups.

Magnetic resonance imaging of the spine was performed at the 8-week final evaluation. The MRIs showed a wide range of pathology severity in the spinal cord at the final end point (figure 1), reflecting the ranges of recovery.

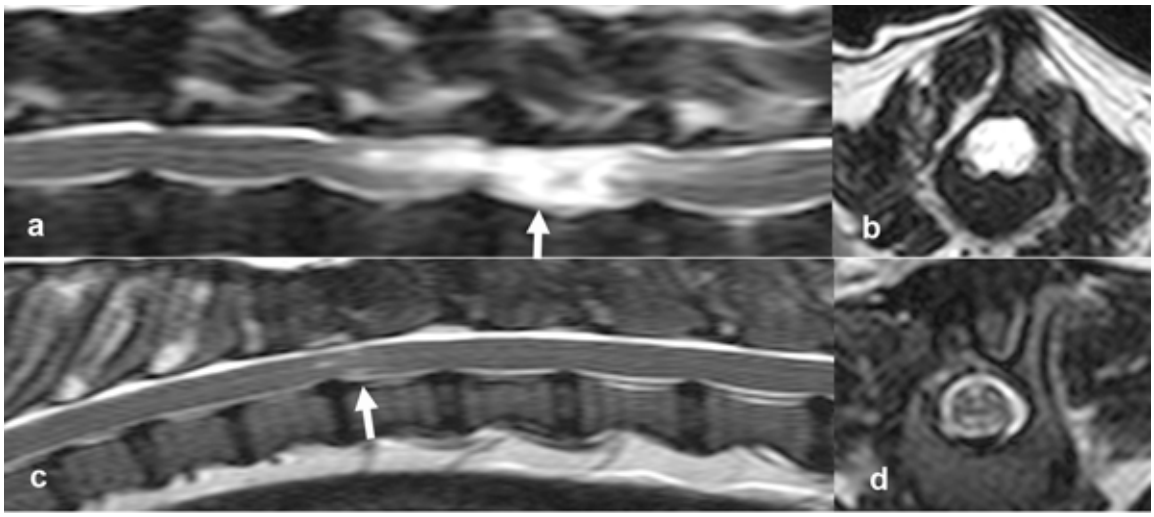

Figure 1: T2 weighted mid sagittal (a and c) and axial (b and d) MR images of the spine of dogs 1 and 5 at the end of the trial (8 weeks post injury). Dog 1 failed to recover pain perception or motor function while dog 5 made an excellent recovery. In both dogs, the site of the original injury is visible as hyperintensity (white arrows) but the degree of injury to the cord at the lesion epicenter (images b and d) is markedly different with complete obliteration of spinal cord parenchyma in dog 1 and only small areas of residual hyperintensity in dog 5. Quantification of the MRI changes is provided in Table 5.

Dog	Group	MRI lesion length*	Maximum % spinal cord compromise
1	GGF2	2.03	100
4	GGF2	1.98	58.22
5	GGF2	0.42	7.1
8	GGF2	0.73	12.4
10	GGF2	0.97	21
2	V	0	0
3	V	NA	NA
6	V	0.53	11
7	V	1.61	64.8
9	V	0	0

Table 5: Measurements of lesion length and cross sectional area on MRI. * MRI lesion length is expressed as a ratio to the length of the second lumbar vertebra to allow comparison between dogs of different sizes. The maximum percentage spinal cord compromise is the lesion cross sectional area expressed as a percentage of the spinal cord cross sectional area. Dog 3 was euthanized so no data is available.

Analysis of myelination at the injury site was attempted through calculation of fractional anisotropy indices from diffusion-weighted images. However, the degree of movement associated with breathing and CSF pulsation, the small size of the spinal cord and the limitations of the diagnostic MRI being used in this study resulted in a large amount of artifact.

Key Research Accomplishments

- Demonstrated that healthy dogs tolerate a single course of 7 days of treatment with human recombinant GGF2 at a dose rate of 0.08mg/kg SC once daily.
- Demonstrated that the same dose of human recombinant GGF2 can be given safely to dogs with severe spinal cord injuries.

Reportable Outcomes

GGF2 can be administered safely to healthy dogs and dogs with spinal cord injury once daily for 7 days. The mean half-life following intravenous administration is 0.23 hours. Repeated rounds of dosing are likely to elicit an immune response to the recombinant protein.

Conclusion

In the first part of this work investigating the use of human recombinant GGF2 in dogs, we demonstrated that it is tolerated in healthy dogs when administered at a dose rate of 0.08mg/kg (equivalent to the 0.24mg/kg rodent dose) once a day SC for 7 days. The half-life ranges from 0.14 to 0.32 hours. Higher doses were associated with injection site pain, and repeated rounds of dosing were associated with hypersensitivity. In the phase 1 trial evaluating GGF2 in dogs with severe spinal cord injuries, we demonstrated that 0.08mg/kg administered SC once a day for 7 days was well tolerated. This study of 10 dogs was not designed to demonstrate efficacy, and if further development of GGF2 as a therapy for spinal cord injury is pursued, a larger phase 2 trial will be designed.

References

1. Olby NJ, De Risio L, Munana KR, Wosar MA, Skeen TM, et al. (2001) Development of a functional scoring system in dogs with acute spinal cord injuries. Am J Vet Res 62: 1624-1628.

2. Olby NJ, Lim JH, Babb K, Bach K, Domaracki C, et al. (2014) Gait scoring in dogs with thoracolumbar spinal cord injuries when walking on a treadmill. BMC Vet Res 10: 58.

3. Atalan G, Barr FJ, Holt PE. Assessment of urinary bladder volume in dogs by use of linear ultrasonographic measurements. Am J Vet Res. 1998;59(1):10-5.

Appendices

Appendix 1: Pharmacokinetic analysis of GGF2 following parenteral and SC administration to hound dogs.

The Determination of GGF2 in Dog Plasma (Li Heparin) by a Quantitative ELISA Method

Acorda Therapeutics Study: GG-SCI-SCIRP

Analytical Site: Acorda Therapeutics, Inc.
420 Saw Mill River Road
Ardsley, NY 10502

Sample Generation: Laboratory of Dr. Natasha Olby

Sponsor: Acorda Therapeutics, Inc.
420 Saw Mill River Road
Ardsley, NY 10502

Study Director: Jennifer Iaci
Acorda Therapeutics, Inc.
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Study Monitor: Andrea Vecchione
Acorda Therapeutics, Inc.
420 Saw Mill River Road
Ardsley, NY 10502

Total Number of Samples Analyzed: 189

Experimental Starting Date: 13 August 2013

Experimental Completion Date: 07 November 2013

Report Date: 21 October 2014

The Determination of GGF2 in Dog Plasma (Li Heparin) by a Quantitative ELISA Method

Acorda Therapeutics Study: GG-SCI-SCIRP

This report describes results from non-GLP laboratory studies conducted in accordance with appropriate Acorda Therapeutics procedures. I have reviewed the study and agree that the data support the conclusions stated herein.

Andrea Vecchione, B.S. Date
Manager-Regulated Studies, BioAnalytics

Donald Button, Ph.D. Date
Director – Cell and Molecular Technologies

The Determination of GGF2 in Dog Plasma (Li Heparin) by a Quantitative ELISA Method

Acorda Therapeutics Study: GG-SCI-SCIRP

Acorda Therapeutics
Research and Development
Study Personnel

Name	Title	Role
Andrey Konovolov	Research Associate	Analyst
Andrea Vecchione	Manager – Regulated Studies, Bioanalytic	Study Monitor, Review
Kathryn Ang, DABT	Toxicologist	Pharmacokinetic Calculations
Donald Button, Ph.D.	Director, Cell and Molecular Technologies	Review

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I. Introduction

This report describes the use of an ELISA-based assay for the determination of GGF2 concentration in lithium heparin dog plasma from the GG-SCI-SCIRP Study. Plasma samples were collected from the animals at multiple time points post injury and stored frozen until completion of the study in the Olby laboratory. They were then shipped to Acorda Therapeutics for assessment of GGF2 plasma levels.

II. Analytical Method and Results

Sample Receipt and Analysis Dates

Plasma samples from GGF2 dosed dogs were shipped from the Olby laboratory on 01 August 2013. Analysis of the samples occurred from 13 August 2013 to 15 August 2013.

Storage of Samples

The plasma samples were received at Acorda Therapeutics, Inc and maintained frozen at approximately -80^{0}C. After analysis, the unused dose solution samples were re-frozen and stored at approximately -80℃.

Analytical Method

The analytical method used in these studies is an adaptation of a quantitative GGF2 ELISA procedure that was validated in rat and monkey plasma at Prevalere Life Sciences, LLC in September 2009 (Prevalere job numbers 174651 and 174652 respectively). A copy of the validated analysis method for rat plasma samples [M08.GGF2.rtp.2, Rev. 3] is located in Appendix A. Thus, the modified quantitative method employed for analysis of dose solutions from the GG_SCI-SCIRP is exactly as described in M08.GGF2.rtp.2, Rev. 3 (Appendix A) with the exception that sample composition was dog plasma (instead of rat plasma) and reference standard and quality control (QC) samples were prepared in naïve dog plasma (instead of rat plasma).

Reference Standard

The following reference standard was used in the analysis:

Name	GGF2
Supplier	CMC ICOS
Lot No.	10-0084
Concentration	4.1 mg/mL
Storage	-80^{0}C

Assay Performance

A summary of the plates can be found in Table 1. Calibration curve standards and quality control (QC) samples prepared by dilution of the reference standard in dog

plasma (Li heparin) were analyzed with the dog plasma study samples as per the Analytical Method. Calibration and QC standard solution concentrations covered the range of 0.50 ng/mL to 32 ng/mL. The lower limit of quantitation (LLOQ) of analyte in the assay reaction is 0.5 ng/mL. Because dose solution samples are diluted by 20-fold prior to assay, the sensitivity of the assay for detection of analyte in dose solution samples is 10 ng/mL. Back-calculated concentrations for standards are provided in Table 2 and the concentrations obtained from the quality control samples are provided in Table 3. The standard back-calculated concentrations and quality control sample results were used to accept each analytical run. A calibrator (0.25 ng/mL in 5% dog plasma) outside of the validated range of the assay was included to serve as an anchor point to facilitate curve fitting.

The quality control samples met the following criteria: the observed concentrations of at least two-thirds of the QC samples (and at least 50% of the replicate QCs at each of three concentration levels) must be within ± 20.0% of their nominal concentrations.

A representative calibration curve with a 4-parameter fit is provided in Figure 1. The X-axis represents the nominal concentration in log (ng/mL) and the Y-axis represents the optical density (OD) units measured at 450 nm. The average R^2 value for the plates was 0.999.

Sample Analysis
Study samples were removed from -80ºC storage and thawed on ice. Samples were diluted 20-fold in Assay Diluent for a final assay concentration of 5% plasma. All samples were analyzed with calibration curve standards and QC standards as per the Analytical Method.

Results
The dilution-corrected concentrations of GGF2 obtained in the dog plasma study samples are provided in summary format in Table 4. The concentration of GGF2 in rat plasma samples ranged from < 10 ng/mL (BLOQ, below the limit of quantitation) to1487.49 ng/mL after the correction for dilution. When possible pharmacokinetic calculations were performed and are provided in Table 5.

Analytical Notes
No analytical notes.

Raw Data
A history of the analysis of samples is located in Acorda Therapeutics notebooks 330 and 348.

III. Tables

Table 1: Plate Summary

Plate	Date	Description
1	8/13/2013	Animals 5 & 6
2	8/14/2013	Animals 1 & 2
3	8/15/2013	Animals 3 & 4

Table 2: Standard Curve Back-Calculated Concentrations

	Nominal [GGF2] (ng/mL)						
	32	**16**	**8**	**4**	**2**	**1**	**0.5**
Plate 1	30.89	16.27	7.77	4.07	2.14	1.03	0.56
	32.89	16.44	7.51	3.97	2.22	0.99	0.41
Mean	31.89	16.35	7.64	4.02	2.18	1.01	0.48
SD	1.42	0.12	0.18	0.07	0.06	0.03	0.10
CV%	4.44	0.74	2.42	1.65	2.53	2.59	21.76
Plate 2	31.75	16.07	8.62	4.53	2.39	1.08	0.42
	31.92	17.24	5.49	4.23	2.23	1.10	0.35
Mean	31.84	16.66	7.06	4.38	2.31	1.09	0.38
SD	0.12	0.82	2.21	0.21	0.11	0.02	0.05
CV%	0.38	4.93	31.31	4.86	4.97	1.69	12.92
Plate 2	32	17	8.2	4.1	2.1	1.4	0.45
	32	16	7.9	3.6	1.9	0.94	0.43
Mean	31.98	16.02	8.07	3.86	2.01	1.17	0.44
SD	0.43	0.71	0.21	0.35	0.15	0.32	0.01
CV%	1.36	4.43	2.65	9.01	7.24	27.60	3.39

Table 3: Quality Control Sample Concentrations

		Acceptance Criteria		
	25%	**30.00**	**7.50**	**1.88**
	-25%	**18.00**	**4.50**	**1.13**
		Nominal Concentrations (ng/mL)		
Plate		**24**	**6**	**1.5**
1		25.19	6.00	1.19
		25.09	5.89	1.08
		22.74	4.97	1.10
		20.97	4.88	1.68
	Mean	23.49	5.44	1.26
	SD	2.03	0.59	0.28
	CV (%)	8.64	10.87	22.15
2		22.85	6.75	1.46
		22.46	6.42	1.35
		20.23	5.74	1.34
		20.24	5.77	1.43
	Mean	21.45	6.17	1.40
	SD	1.41	0.50	0.06
	CV (%)	6.56	8.07	4.30
3		23.77	5.86	1.18
		21.92	5.84	1.19
		22.07	5.45	1.00
		21.86	5.46	1.02
	Mean	22.41	5.65	1.10
	SD	0.91	0.23	0.10
	CV (%)	4.07	4.00	9.23

Table 4: Sample Results

Sample Identifier	Treatment Date	Dog Name	Dog Number	Sampling Timepoint (hr)	Dose (mg/kg)	Route	Plasma Concentration (ng/mL)
1-0 4/03	4/3/2013	Dahlia	1	baseline	0.24	SC	BLOQ
1-1 4/03	4/3/2013	Dahlia	1	0.25	0.24	SC	BLOQ
1-2 4/03	4/3/2013	Dahlia	1	0.5	0.24	SC	BLOQ
1-3 4/03	4/3/2013	Dahlia	1	1	0.24	SC	BLOQ
1-4 4/03	4/3/2013	Dahlia	1	2	0.24	SC	BLOQ
1-5 4/03	4/3/2013	Dahlia	1	4	0.24	SC	BLOQ
1-6 4/03	4/3/2013	Dahlia	1	6	0.24	SC	BLOQ
1-7 4/04	4/3/2013	Dahlia	1	24	0.24	SC	BLOQ
1-8 4/10	4/10/2013	Dahlia	1	baseline	0.24	SC	BLOQ
1-9 4/10	4/10/2013	Dahlia	1	0.25	0.24	SC	BLOQ
1-10 4/10	4/10/2013	Dahlia	1	0.5	0.24	SC	BLOQ
1-11 4/10	4/10/2013	Dahlia	1	1	0.24	SC	BLOQ
1-12 4/10	4/10/2013	Dahlia	1	2	0.24	SC	BLOQ
1-13 4/10	4/10/2013	Dahlia	1	4	0.24	SC	BLOQ
1-14 4/10	4/10/2013	Dahlia	1	6	0.24	SC	BLOQ
1-15 4/11	4/10/2013	Dahlia	1	24	0.24	SC	BLOQ
1-0 5/15	5/15/2013	Dahlia	1	baseline	0.08	IV	BLOQ
1-1 5/15	5/15/2013	Dahlia	1	0.0833	0.08	IV	1487.49
1-2 5/15	5/15/2013	Dahlia	1	0.25	0.08	IV	274.09
1-3 5/15	5/15/2013	Dahlia	1	0.5	0.08	IV	84.24
1-4 5/15	5/15/2013	Dahlia	1	1	0.08	IV	11.052
1-5 5/15	5/15/2013	Dahlia	1	2	0.08	IV	BLOQ
1-6 5/15	5/15/2013	Dahlia	1	4	0.08	IV	BLOQ
1-7 5/15	5/15/2013	Dahlia	1	6	0.08	IV	BLOQ
1-8 5/16	5/15/2013	Dahlia	1	24	0.08	IV	BLOQ
1-9 5/21*	5/21/2013	Dahlia	1	baseline	0.08	IV	BLOQ
1-10 5/21	5/21/2013	Dahlia	1	0.5	0.08	IV	BLOQ
1-11 5/21	5/21/2013	Dahlia	1	60	0.08	IV	BLOQ
1-12 5/21	5/21/2013	Dahlia	1	2	0.08	IV	BLOQ
1-13 5/21	5/21/2013	Dahlia	1	4	0.08	IV	BLOQ
1-14 5/21	5/21/2013	Dahlia	1	6	0.08	IV	BLOQ
1-15 5/21	5/21/2013	Dahlia	1	24	0.08	IV	BLOQ

*did not receive full dose

Table 4: Sample Results Cont.

Sample Identifier	Treatment Date	Dog Name	Dog Number	Sampling Timepoint (hr)	Dose (mg/kg)	Route	Plasma Concentration (ng/mL)
2-0 4/03	4/3/2013	Clover	2	baseline	0.24	IV	BLOQ
2-1 4/03	4/3/2013	Clover	2	0.0833	0.24	IV	156.27
2-2 4/03	4/3/2013	Clover	2	0.25	0.24	IV	64.36
2-3 4/03	4/3/2013	Clover	2	0.5	0.24	IV	20.72
2-4 4/03	4/3/2013	Clover	2	1	0.24	IV	BLOQ
2-5 4/03	4/3/2013	Clover	2	2	0.24	IV	BLOQ
2-6 4/03	4/3/2013	Clover	2	4	0.24	IV	BLOQ
2-7 4/03	4/3/2013	Clover	2	6	0.24	IV	BLOQ
2-8 4/04	4/3/2013	Clover	2	24	0.24	IV	BLOQ
2-9 4/10	4/10/2013	Clover	2	baseline	0.24	IV	BLOQ
2-10 4/10	4/10/2013	Clover	2	0.0833	0.24	IV	409.31
2-11 4/10	4/10/2013	Clover	2	0.25	0.24	IV	144.65
2-12 4/10	4/10/2013	Clover	2	0.5	0.24	IV	55.23
2-13 4/10	4/10/2013	Clover	2	1	0.24	IV	21.77
2-14 4/10	4/10/2013	Clover	2	2	0.24	IV	BLOQ
2-15 4/10	4/10/2013	Clover	2	4	0.24	IV	BLOQ
2-16 4/10	4/10/2013	Clover	2	6	0.24	IV	BLOQ
2-17 4/11	4/10/2013	Clover	2	24	0.24	IV	BLOQ
2-0 5/15	5/15/2013	Clover	2	baseline	0.08	SC	BLOQ
2-1 5/15	5/15/2013	Clover	2	0.25	0.08	SC	BLOQ
2-2 5/15	5/15/2013	Clover	2	0.5	0.08	SC	BLOQ
2-3 5/15	5/15/2013	Clover	2	1	0.08	SC	BLOQ
2-4 5/15	5/15/2013	Clover	2	2	0.08	SC	BLOQ
2-5 5/15	5/15/2013	Clover	2	4	0.08	SC	BLOQ
2-6 5/15	5/15/2013	Clover	2	6	0.08	SC	BLOQ
2-7 5/16	5/15/2013	Clover	2	24	0.08	SC	BLOQ
2-8 5/21	5/21/2013	Clover	2	baseline	0.08	SC	BLOQ
2-9 5/21	5/21/2013	Clover	2	0.25	0.08	SC	BLOQ
2-10 5/21	5/21/2013	Clover	2	0.5	0.08	SC	BLOQ
2-11 5/21	5/21/2013	Clover	2	1	0.08	SC	BLOQ
2-12 5/21	5/21/2013	Clover	2	2	0.08	SC	BLOQ
2-13 5/21	5/21/2013	Clover	2	4	0.08	SC	BLOQ
2-14 5/21	5/21/2013	Clover	2	6	0.08	SC	BLOQ
2-15 5/22	5/21/2013	Clover	2	24	0.08	SC	BLOQ

Table 4: Sample Results Cont.

Sample Identifier	Treatment Date	Dog Name	Dog Number	Sampling Timepoint (hr)	Dose (mg/kg)	Route	Plasma Concentration (ng/mL)
3-0 4/03	4/3/2013	Magnolia	3	baseline	0.24	SC	BLOQ
3-1 4/03	4/3/2013	Magnolia	3	0.25	0.24	SC	BLOQ
3-2 4/03	4/3/2013	Magnolia	3	0.5	0.24	SC	BLOQ
3-3 4/03	4/3/2013	Magnolia	3	1	0.24	SC	BLOQ
3-4 4/03	4/3/2013	Magnolia	3	2	0.24	SC	BLOQ
3-5 4/03	4/3/2013	Magnolia	3	4	0.24	SC	BLOQ
3-6 4/03	4/3/2013	Magnolia	3	6	0.24	SC	BLOQ
3-7 4/04	4/3/2013	Magnolia	3	24	0.24	SC	BLOQ
3-8 4/10	4/10/2013	Magnolia	3	baseline	0.24	SC	BLOQ
3-9 4/10	4/10/2013	Magnolia	3	0.25	0.24	SC	BLOQ
3-10 4/10	4/10/2013	Magnolia	3	0.5	0.24	SC	BLOQ
3-11 4/10	4/10/2013	Magnolia	3	1	0.24	SC	BLOQ
3-12 4/10	4/10/2013	Magnolia	3	2	0.24	SC	BLOQ
3-13 4/10	4/10/2013	Magnolia	3	4	0.24	SC	BLOQ
3-14 4/10	4/10/2013	Magnolia	3	6	0.24	SC	BLOQ
3-15 4/11	4/10/2013	Magnolia	3	24	0.24	SC	BLOQ
3-0 5/15	5/15/2013	Magnolia	3	baseline	0.08	SC	BLOQ
3-1 5/15	5/15/2013	Magnolia	3	0.25	0.08	SC	BLOQ
3-2 5/15	5/15/2013	Magnolia	3	0.5	0.08	SC	BLOQ
3-3 5/15	5/15/2013	Magnolia	3	1	0.08	SC	BLOQ
3-4 5/15	5/15/2013	Magnolia	3	2	0.08	SC	BLOQ
3-5 5/15	5/15/2013	Magnolia	3	4	0.08	SC	BLOQ
3-6 5/15	5/15/2013	Magnolia	3	6	0.08	SC	BLOQ
3-7 5/16	5/15/2013	Magnolia	3	24	0.08	SC	BLOQ
3-8 5/21	5/21/2013	Magnolia	3	baseline	0.08	SC	BLOQ
3-9 5/21	5/21/2013	Magnolia	3	0.25	0.08	SC	BLOQ
3-10 5/21	5/21/2013	Magnolia	3	0.5	0.08	SC	BLOQ
3-11 5/21	5/21/2013	Magnolia	3	1	0.08	SC	BLOQ
3-12 5/21	5/21/2013	Magnolia	3	2	0.08	SC	BLOQ
3-13 5/21	5/21/2013	Magnolia	3	4	0.08	SC	BLOQ
3-14 5/21	5/21/2013	Magnolia	3	6	0.08	SC	BLOQ
3-15 5/22	5/21/2013	Magnolia	3	24	0.08	SC	BLOQ

Table 4: Sample Results Cont.

Sample Identifier	Treatment Date	Dog Name	Dog Number	Sampling Timepoint (hr)	Dose (mg/kg)	Route	Plasma Concentration (ng/mL)
4-0 4/04	4/4/2013	Cappie	4	baseline	0.24	IV	BLOQ
4-1 4/04	4/4/2013	Cappie	4	0.0833	0.24	IV	275.36
4-2 4/04	4/4/2013	Cappie	4	0.25	0.24	IV	125.08
4-3 4/04	4/4/2013	Cappie	4	0.5	0.24	IV	60.01
4-4 4/04	4/4/2013	Cappie	4	1	0.24	IV	23.87
4-5 4/04	4/4/2013	Cappie	4	2	0.24	IV	BLOQ
4-6 4/04	4/4/2013	Cappie	4	4	0.24	IV	BLOQ
4-7 4/04	4/4/2013	Cappie	4	6	0.24	IV	BLOQ
4-8 4/05	4/4/2013	Cappie	4	24	0.24	IV	BLOQ
4-9 4/11	4/11/2013	Cappie	4	baseline	0.24	IV	BLOQ
4-10 4/11	4/11/2013	Cappie	4	0.0833	0.24	IV	312.22
4-11 4/11	4/11/2013	Cappie	4	0.25	0.24	IV	111.74
4-12 4/11	4/11/2013	Cappie	4	0.5	0.24	IV	31.13
4-13 4/11	4/11/2013	Cappie	4	1	0.24	IV	19.25
4-14 4/11	4/11/2013	Cappie	4	2	0.24	IV	BLOQ
4-15 4/11	4/11/2013	Cappie	4	4	0.24	IV	BLOQ
4-16 4/11	4/11/2013	Cappie	4	6	0.24	IV	BLOQ
4-17 4/12	4/11/2013	Cappie	4	24	0.24	IV	BLOQ
4-0 5/16	5/16/2013	Cappie	4	baseline	0.08	SC	BLOQ
4-1 5/16	5/16/2013	Cappie	4	0.25	0.08	SC	BLOQ
4-2 5/16	5/16/2013	Cappie	4	0.5	0.08	SC	BLOQ
4-3 5/16	5/16/2013	Cappie	4	1	0.08	SC	BLOQ
4-4 5/16	5/16/2013	Cappie	4	2	0.08	SC	BLOQ
4-5 5/16	5/16/2013	Cappie	4	4	0.08	SC	BLOQ
4-6 5/16	5/16/2013	Cappie	4	6	0.08	SC	BLOQ
4-7 5/17	5/16/2013	Cappie	4	24	0.08	SC	BLOQ
4-8 5/22	5/22/2013	Cappie	4	baseline	0.08	SC	BLOQ
4-9 5/22	5/22/2013	Cappie	4	0.25	0.08	SC	BLOQ
4-10 5/22	5/22/2013	Cappie	4	0.5	0.08	SC	BLOQ
4-11 5/22	5/22/2013	Cappie	4	1	0.08	SC	BLOQ
4-12 5/22	5/22/2013	Cappie	4	2	0.08	SC	BLOQ
4-13 5/22	5/22/2013	Cappie	4	4	0.08	SC	BLOQ
4-14 5/22	5/22/2013	Cappie	4	6	0.08	SC	BLOQ
4-15 5/23	5/22/2013	Cappie	4	24	0.08	SC	BLOQ

Table 4: Sample Results Cont.

Sample Identifier	Treatment Date	Dog Name	Dog Number	Sampling Timepoint (hr)	Dose (mg/kg)	Route	Plasma Concentration (ng/mL)
5-0 4/04	4/4/2013	Max	5	baseline	0.24	SC	BLOQ
5-1 4/04	4/4/2013	Max	5	0.25	0.24	SC	BLOQ
5-2 4/04	4/4/2013	Max	5	0.5	0.24	SC	BLOQ
5-3 4/04	4/4/2013	Max	5	1	0.24	SC	BLOQ
5-4 4/04	4/4/2013	Max	5	2	0.24	SC	BLOQ
5-5 4/04	4/4/2013	Max	5	4	0.24	SC	BLOQ
5-6 4/04	4/4/2013	Max	5	6	0.24	SC	BLOQ
5-7 4/05	4/4/2013	Max	5	24	0.24	SC	BLOQ
5-8 4/11	4/11/2013	Max	5	baseline	0.24	SC	175.19
5-9 4/11	4/11/2013	Max	5	0.25	0.24	SC	94.58
5-10 4/11	4/11/2013	Max	5	0.5	0.24	SC	40.35
5-11 4/11	4/11/2013	Max	5	1	0.24	SC	BLOQ
5-12 4/11	4/11/2013	Max	5	2	0.24	SC	BLOQ
5-13 4/11	4/11/2013	Max	5	4	0.24	SC	BLOQ
5-14 4/11	4/11/2013	Max	5	6	0.24	SC	BLOQ
5-15 4/12	4/11/2013	Max	5	24	0.24	SC	BLOQ
5-0 5/16	5/16/2013	Max	5	baseline	0.08	SC	BLOQ
5-1 5/16	5/16/2013	Max	5	0.25	0.08	SC	BLOQ
5-2 5/16	5/16/2013	Max	5	0.5	0.08	SC	BLOQ
5-3 5/16	5/16/2013	Max	5	1	0.08	SC	BLOQ
5-4 5/16	5/16/2013	Max	5	2	0.08	SC	BLOQ
5-5 5/16	5/16/2013	Max	5	4	0.08	SC	BLOQ
5-6 5/16	5/16/2013	Max	5	6	0.08	SC	BLOQ
5-7 5/17	5/16/2013	Max	5	24	0.08	SC	BLOQ

Table 4: Sample Results Cont.

Sample Identifier	Treatment Date	Dog Name	Dog Number	Sampling Timepoint (hr)	Dose (mg/kg)	Route	Plasma Concentration (ng/mL)
6-0 4/04	4/4/2013	Evan	6	baseline	0.24	SC	BLOQ
6-1 4/04	4/4/2013	Evan	6	0.25	0.24	SC	BLOQ
6-2 4/04	4/4/2013	Evan	6	0.5	0.24	SC	BLOQ
6-3 4/04	4/4/2013	Evan	6	1	0.24	SC	BLOQ
6-4 4/04	4/4/2013	Evan	6	2	0.24	SC	BLOQ
6-5 4/04	4/4/2013	Evan	6	4	0.24	SC	BLOQ
6-6 4/04	4/4/2013	Evan	6	6	0.24	SC	BLOQ
6-7 4/05	4/4/2013	Evan	6	24	0.24	SC	BLOQ
6-8 4/11	4/11/2013	Evan	6	baseline	0.24	SC	BLOQ
6-9 4/11	4/11/2013	Evan	6	0.25	0.24	SC	BLOQ
6-10 4/11	4/11/2013	Evan	6	0.5	0.24	SC	BLOQ
6-11 4/11	4/11/2013	Evan	6	1	0.24	SC	BLOQ
6-12 4/11	4/11/2013	Evan	6	2	0.24	SC	BLOQ
6-13 4/11	4/11/2013	Evan	6	4	0.24	SC	BLOQ
6-14 4/11	4/11/2013	Evan	6	6	0.24	SC	BLOQ
6-15 4/12	4/11/2013	Evan	6	24	0.24	SC	BLOQ
6-0 5/16	5/16/2013	Evan	6	baseline	0.08	IV	BLOQ
6-1 5/16	5/16/2013	Evan	6	0.0833	0.08	IV	BLOQ
6-2 5/16	5/16/2013	Evan	6	0.25	0.08	IV	BLOQ
6-3 5/16	5/16/2013	Evan	6	0.5	0.08	IV	BLOQ
6-4 5/16	5/16/2013	Evan	6	1	0.08	IV	BLOQ
6-5 5/16	5/16/2013	Evan	6	2	0.08	IV	BLOQ
6-6 5/16	5/16/2013	Evan	6	4	0.08	IV	BLOQ
6-7 5/16	5/16/2013	Evan	6	6	0.08	IV	BLOQ
6-8 5/17	5/16/2013	Evan	6	24	0.08	IV	BLOQ
6-9 5/22	5/22/2013	Evan	6	baseline	0.08	SC	BLOQ
6-10 5/22	5/22/2013	Evan	6	0.25	0.08	SC	BLOQ
6-11 5/22	5/22/2013	Evan	6	0.5	0.08	SC	BLOQ
6-12 5/22	5/22/2013	Evan	6	1	0.08	SC	BLOQ
6-13 5/22	5/22/2013	Evan	6	2	0.08	SC	BLOQ
6-14 5/22	5/22/2013	Evan	6	4	0.08	SC	BLOQ
6-15 5/22	5/22/2013	Evan	6	6	0.08	SC	BLOQ
6-16 5/23	5/22/2013	Evan	6	24	0.08	SC	BLOQ

Table 5: Pharmacokinetic Calculations

	route																	
	IV												SC					
	Dose_Number												Dose_Number					
	1						2						1					
Dog_Name	t ½ (h)	Tmax (h)	Cmax (ng/mL)	Tlast (h)	AUClast (h*ng/mL)	AUCall (h*ng/mL)	t ½ (h)	Tmax (h)	Cmax (ng/mL)	Tlast (h)	AUClast (h*ng/mL)	AUCall (h*ng/mL)	t ½ (h)	Tmax (h)	Cmax (ng/mL)	Tlast (h)	AUClast (h*ng/mL)	AUCall (h*ng/mL)
Cappie	0.32	0.083	275.36	1	89	101	0.24	0.083	312.22	1	79	88	-	-	-	-	-	-
Clover	0.14	0.083	156.27	1	36	41	0.29	0.083	409.31	1	107	118	-	-	-	-	-	-
Dahlia	0.16	0.083	1487.49	1	277	283		-	-	-	-	-	-	-	-	-	-	-
Max	-	-	-	-	-	-	-	-	-	-	-	-	0.24	0	175.19	1	51	61

IV. Figures

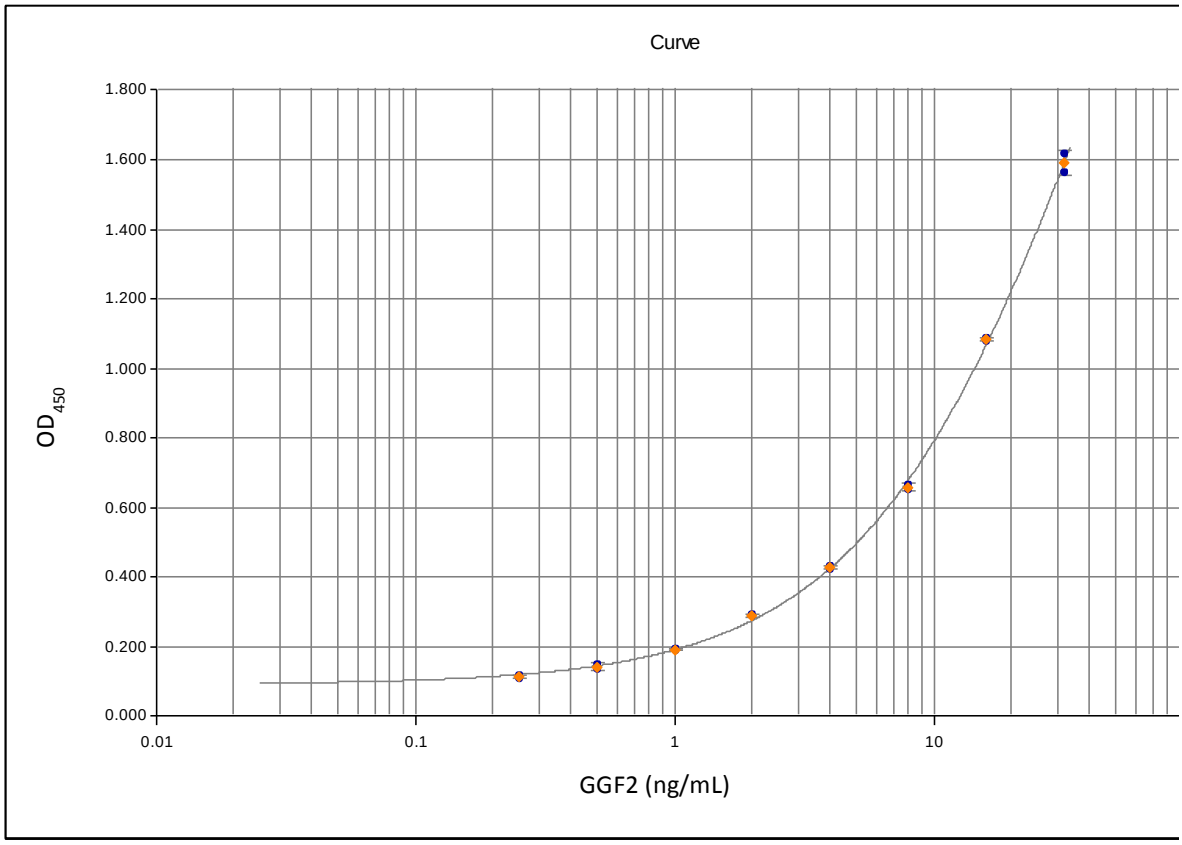

Figure 1: Representative Standard Curve of GGF2 in 5% dog plasma, Plate 1. X-axis nominal GGF2 concentration (ng/mL), Y-axis is optical density measured at 450 nm (OD_{450}).

V. Appendix A: Immunoassay Method

Prevalere Life Sciences, LLC

Immunoassay Method

Method No.:	**M08.GGF2.rtp.2**	**Rev. 3**
Title:	**Determination of GGF2 in Rat Plasma (Li Heparin) by Quantitative ELISA.**	
Scope:	**Assaying of Rat Plasma Samples**	
Responsibility:	**Immunoassay Technologies Lab Personnel**	
Revision(s):	**See Appendix A**	
Revised By:	_____	**Date:** 10/20/09
Reviewed By:	Kateri A. Limmer	**Date:** 10/20/09

Document references: **Central Library for ELISA Reagent Methods**
Central Library for ELISA Buffer Methods

Alternative preparations are allowed and must be fully documented.
Equivalent substitutions may be made.
Adjustable pipettes are used in this method.
Weights and volumes may be scaled as needed.
Alternative volumes of solutions and quality control samples may be prepared as needed.
Please refer to the "Validation Summary" for information such as stability, cross-validation, equipment substitutions and other associated details.
Upgrades to software may be used for all instruments employed.

Method Summary:

This Enzyme Linked ImmunoSorbent Assay (ELISA) is designed to quantify GGF2 in Rat Plasma (Li Heparin). In this ELISA method, a goat anti-GGF2 is diluted in Coating Buffer and immobilized onto a 96-well microtiter sample plate. The plate is washed, and all un-adsorbed sites are blocked with the addition of Block Buffer. Analytes (standards, quality control samples (QCs), and samples) are diluted 1:20 with Diluent Buffer. Appropriate subsequent dilutions of each sample and dilution integrity QC are made with 5% matrix in Diluent Buffer so that they may fall into the linear range of the standard curve. Diluted samples are dispensed into the 96-well microtiter sample plate followed by incubation for approximately 1 hour. Plates are then washed and an anti-NRGβ1 Biotin Conjugate is added and incubated for approximately 1 hour. Plates are then washed and a Streptavidin-HRP Conjugate is added and incubated for approximately 30 minutes. After the final wash step, a tetramethylbenzidine (TMB) peroxidase Substrate Solution is added and incubated for approximately 5 minutes if Biotin used in preparation of Working Detector Solution is of Lot EEE0509021, and 15 minutes if Biotin used in preparation of Working Detector Solution is of Lot EEE030808. The reaction is stopped with a Phosphoric Acid Stop Solution. Color develops in proportion to the amount of GGF2 in the sample. Plates are read on a plate reader using two filters (450 nm for detection and 620 nm for background). GGF2 concentrations are determined on a standard curve obtained by plotting optical density (OD) versus concentration using a four-parameter logistic curve-fitting program. The calibration curve range of this method in Rat Plasma (Li Heparin) is 32.0 ng/mL – 0.500 ng/mL in 5% Matrix (640 ng/mL – 10.0 ng/mL in 100% Matrix). A calibrator outside the validated range of the assay at 0.250 ng/mL in 5% Matrix may be included to serve as an anchor point to facilitate curve-fitting. The anchor point is removed or retained based on the best fit of the curve (i.e., the highest number of standards read within defined accuracy, %RE.)

Materials and Equipment:

96 well Nunc-Immuno Module, C8 Starwell MaxiSorp™ microtiter plates (Nunc, # 441653 or equivalent)

Acetate microtiter plate sealer (Thermo Labsystems # 3501 or equivalent)

Plate washer to dispense and aspirate at 300 µL wash volume (Bio-Tek ELx405 Select or equivalent)

Automated dilutor (Bio-Tek Precision™ or equivalent)

Automated dilutor software (Bio-Tek Precision Power™ ver. 2.0 or higher)

Plate reader with reading capabilities at 450 nm and 620 nm wavelengths (Bio-Tek PowerWave HT™ or equivalent)

Computer with 4-parameter logistic curve fitting program (Gen5 Secure Software ver.1.08 or higher)

Single- and multi-channel adjustable volume manual pipettors (Nichiryo or equivalent)

Microtiter plate orbital shaker (Lab-Line # 4625 or equivalent)

pH meter (Corning M530 or equivalent)

Analytical balance (Ohaus Explorer or equivalent)

Biologics:

Anti-human NRG1 Isoform GGF2 Antibody. Supplied by R&D systems #AF2015. Store at 2-8°C for short-term storage. Aliquot and store at -70°C or colder for long-term storage.

GGF2. Supplied by Fulcrum. Aliquot and store at -70°C or colder.

Biotinylated anti-human NRG1- ß1. Supplied by R&D systems. DuoSet Detection Antibody #DY377 Part No.841080 or #BAF377. Aliquot and store at -70°C or colder.

Streptavidin HRP. Supplied by Southern Biotech # 7100-05. Store at 2-8°C.

Bovine Serum Albumin (BSA). Supplied by Millipore # 81-003-3 or equivalent. Store at 2-8°C.

Sucrose. Supplied by Sigma #S9378 or equivalent. Store at ambient temperature.

Sprague-Dawley Rat Plasma (Lithium Heparin). Supplied by Biochemed or equivalent. Store at -70°C or colder.

Goat Serum. Supplied by Biochemed or equivalent. Store at -70°C or colder.

Reagents:

Coating Buffer, pH 9.6 (50 mM Sodium Carbonate)	Prepared per ELISA Buffer Method EB-01
Wash Buffer, pH 8.0 (1X TBST)	Prepared per ELISA Buffer Method EB-34
Block Buffer (1XTBS/ 1% BSA/ 5% Sucrose)	Prepared per ELISA Buffer Method EB-59
Diluent Buffer (1X TBS/ 1% BSA)	Prepared per ELISA Buffer Method EB-48
Working Coat Solution	Dilute anti-human NRG1 Isoform GGF2 Ab stock solution with Coating Buffer to a final working concentration of 1 µg/mL. Prepare fresh daily and discard after use.
Working Detector Solution	Dilute biotinylated anti-human NRG1-ß1 stock solution with 2% normal goat serum in Diluent Buffer to a final working concentration of 200 ng/mL. Prepare fresh daily and discard after use.
Working Conjugate Solution	Dilute Streptavidin HRP stock solution 1:30,000 in Diluent Buffer. Prepare fresh daily and discard after use.
Working Substrate Solution: TMB Microwell Peroxidase Substrate System. (KPL, # 50-76-00)	Prepare a 50:50 solution. (TMB Peroxidase Substrate: Peroxidase Substrate Solution B). Prepare fresh daily and discard after use.
Phosphoric Acid Stop Solution (1M H_3PO_4)	Prepared per ELISA Buffer Method EB-06

Calibration Standards and Quality Control Samples for GGF2 in Rat Plasma (Li Heparin):

The standard stock (3.5 mg/mL for example) is diluted in 100% rat plasma (Li Heparin) at 20X concentrations as described below:

20X Standard Curve Sample Preparation

Stock Identity at 20X	Concentration at 20X (ng/mL)	Volume of Working Stock (µL)	Volume of 100% Matrix (µL)	Identity at 1X	Concentration at 1X (ng/mL)
A	100,000	30.0 of Primary Stock	1020	--	--
B	10,000	40.0 of A	360	--	--
B1	640	192 of B	2808	**S1**	32.0
B2	320	1375 of B1	1375	**S2**	16.0
B3	160	1375 of B2	1375	**S3**	8.00
B4	80.0	1375 of B3	1375	**S4**	4.00
B5	40.0	1375 of B4	1375	**S5**	2.00
B6	20.0	1375 of B5	1375	**S6**	1.00
B7	10.0	1375 of B6	1375	**S7**	0.500
B8	5.0	1375 of B7	1375	**S8+**	0.250

+ The S8 standard is a calibrator outside the validated range of the assay that may serve as an anchor point to facilitate curve fitting. Anchor points are only used for curve fitting and are not evaluated in curve assessments.

20X Quality Control Sample Preparation

Stock Identity	Concentration at 20X (ng/mL)	Volume of Working Stock (µL)	Volume of 100% Matrix (µL)	Identity at 1X	Concentration at 1X (ng/mL)
C	100,000	30.0 of Primary Stock	1020	--	--
D	10,000	40.0 of C	360	--	--
D1	480	192 of D	3808	**High QC**	24.0
D2	120	1000 of D1	3000	**Mid QC**	6.00
D3	30.0	875 of D2	2625	**Low QC**	1.50
D4	640	64.0 of D	936	**ULOQ***	32.0
D5	10.0	270 of D3	540	**LLOQ***	0.500

*ULOQ and LLOQ will be used for Precision and Accuracy runs only.

Procedures:

1. Dispense 100 µL per well of the Working Coat solution to the wells of a 96 well Nunc-Immuno Module, C8 Starwell MaxiSorp™ microtiter plate. Seal and incubate plate at ambient temperature for approximately 1 hour.

2. Remove solutions from wells by aspirating with a plate washer. Wash and aspirate each well with approximately 300 µL of Wash Buffer 3 times. The washer is programmed to have a soaking time of 10 seconds. Tap out the excess liquid from the wells.

3. To block un-adsorbed sites, dispense 300 µL per well of Block Buffer to the wells of a pre-coated microtiter plate. Incubate at ambient temperature for a minimum of 1 hour and not more than 3 hours with non-vigorous shaking.

4. Remove solutions from wells by aspirating with a plate washer. Wash and aspirate each well with approximately 300 µL of Wash Buffer 3 times. The washer is programmed to have a soaking time of 10 seconds. Tap out the excess liquid from the wells.

5. Prepare and dilute standards (20X), quality control samples (dilution integrity and 20X), blanks, and samples 1:20 with Diluent Buffer. Make appropriate subsequent dilutions of each sample and dilution integrity QC with 5% matrix in Diluent Buffer so that they may fall into the linear range of the standard curve. Dispense 100 µL into appropriate wells of the sample microtiter plate. Analyze samples and standards in duplicate, quality control samples in quadruplicate, (with two independent determinations), and 6 replicates of a dilution integrity QC that covers the maximum dilution on the plate beyond the minimum required dilution. Seal and incubate at ambient temperature for approximately 1 hour with non-vigorous shaking.

6. Immediately following step 5 above, prepare Working Detector Solution. Mix by inversion for a minimum of 1 hour at ambient temperature.

7. Remove solutions from wells by aspirating with a plate washer. Wash and aspirate each well with approximately 300 µL of Wash Buffer 3 times. The washer is programmed to have a soaking time of 10 seconds. Turn the plate 180° and repeat. Tap out the excess liquid from the wells.

8. Dispense 100 µL per well of the Working Detector Solution. Cover and incubate at ambient temperature for approximately 1 hour with non-vigorous shaking.

9. Remove solutions from wells by aspirating with a plate washer. Wash and aspirate each well with approximately 300 µL of Wash Buffer 3 times. The washer is programmed to have a soaking time of 10 seconds. Turn the plate 180° and repeat. Tap out the excess liquid from the wells.

10. Dispense 100 µL per well of the Working Conjugate Solution. Cover and incubate at ambient temperature for approximately 30 minutes with non-vigorous shaking.

11. Remove solutions from wells by aspirating with a plate washer. Wash and aspirate each well with approximately 300 µL of Wash Buffer 3 times. The washer is programmed to have a soaking time of 10 seconds. Turn the plate 180° and repeat. Tap out the excess liquid from the wells.

12. Add 100 µL per well of the Working TMB Substrate Solution. Incubate for approximately 5 minutes if Biotin used in preparation of Working Detector Solution is of Lot EEE0509021, and 15 minutes if Biotin used in preparation of Working Detector Solution is of Lot EEE030808.

13. Dispense 100 µL per well of the Phosphoric Acid Stop Solution to stop reaction. Note: Non-vigorous shaking may be performed, but is not necessary post stop solution dispense. Read OD on a plate reader using two filters (450 nm for detection and 620 nm for background). Plates may be read up to 30 minutes after the addition of stop solution.

Calculations and Analysis:

Sample concentrations are determined by back-calculating from standards that have been entered into a 4-parameter logistic curve-fitting program.

Appendix A:

Revision 1:

1. Under Reagents, changed statement about Working Coat Solution.

Revision 2:

1. Removed Dilution Integrity QC Preparation Table and updated procedure Step 5 to reflect lab practice of diluting the dilution integrity QC.

Revision 3:

1. Indicated Sprague-Dawley as the strain of the rat plasma.
2. Under Biologics, updated storage of anti-human NRG1 Isoform GGF2 Antibody.

VI. Amendments

No amendments have been issued for this report as of the report date.